STEROID HORMONES AND TRANSCRIPTION FACTORS IN YOLK PROTEIN SYNTHESIS OF *MUSCA DOMESTICA*

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Steroid hormones and transcription factors in yolk protein synthesis of *Musca domestica*

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1 Summary

Synthesis of yolk proteins (YP) is regulated by sex-specific proteins encoded by the gene *doublesex* (*dsx*) in the Drosophila fat body. In females, the basal transcription rate is enhanced by the binding of the female-specific protein DSX^F to the enhancer of the *yp* genes, whereas in males, YP expression is completely repressed by the male-specific protein DSX^M. Synthesis of yolk proteins in *Musca domestica*, on the other hand, appears to be regulated by sex-specific differences in the concentration of ecdysteroids. In females, the level of YP in the hemolymph follows a cycle that correlates with the cycling of ecdysteroid levels, whereas in males, the ecdysteroid titre is constantly low, and no YP are produced. Injection of 20-hydroxy-ecdysone (20E) into males induces YP synthesis. However, there are some indications that there must be additional factors involved in the regulation of YP expression in Musca. Males do not respond as well to injection of 20E as females, and ovaryectomized females continue to express YP, though their ecdysteroid titer drops to a very low, male-like level.

The objective of my thesis work was to investigate whether sex-specific transcription factors also contribute to the regulation of YP synthesis in *Musca domestica*. I analyzed two Musca strains in which males produce small amounts of YP, and found that these males do not have elevated ecdysteroid levels. This fact further supports the notion that additional factors take part in the regulation of YP synthesis in Musca. We then tested if a *dsx* homologue is present in Musca, and whether it is involved in the control of YP expression. We were able to identify a *dsx* homologue, *Md-dsx*, which is sex-specifically spliced and gives rise to two different proteins, the female form Md-DSX^F and the male form Md-DSX^M.

Next, we examined whether regulation of *Md-dsx* is based on a conserved mechanism. We identified, in the primary transcript of *Md-dsx*, *dsx* repeat elements (dsxRE), which are also present in *dsx* homologues of *Drosophila melanogaster*, *Megaselia scalaris* and *Bactrocera tryoni*. In Drosophila, binding of the protein complex TRA/TRA2 to these elements imposes the female splice mode. We therefore tested whether homologues of *tra* and *tra2* are present in Musca. We identified a Musca homologue of *tra2*, and we demonstrated that *Md-tra2* is necessary for the female splicing of *Md-dsx*, and essential for female development. Recently, we were also able to identify a putative *tra* homologue.

In standard males, only the male isoform of *Md-dsx* is expressed. However, in the YP expressing males, substantial levels of *Md-dsx*^{F} transcripts can be detected. Also, expression of Md-DSX^F in standard males carrying an inducible transgene can promote the production of YP. This suggested to us that YP synthesis in Musca is controlled by a concerted action of Md-DSX proteins and ecdysteroids. We propose that presence of Md-DSX^F in female fat body cells increases the competence of the YP genes to respond

to activation by ecdysteroids, while in males, the threshold for activation is markedly increased by the presence of Md-DSX^M. Hormones, on the other hand, serve different purposes. First, they synchronize YP synthesis with onset of vitellogenesis in oocyte development. Second, hormones adjust YP synthesis to environmental conditions such as availability of food resources and egg-laying substrate.

The use of autonomous competence factors for sex- and tissue-specificity, combined with the use of non-autonomous factors that respond to extrinsic conditions, may be a common mechanism for the control of YP synthesis in insects. However, the contribution of these two systems may vary, depending on the mode of ovarian development. In Musca, hormones appear to play a more distinct role than in Drosophila, because oogenesis in Musca is not a continuous process but rather occurs in cycles, and YP synthesis thus needs to be coordinated with oocyte development.

2 Zusammenfassung

Die Dotterproteinsynthese im Fettkörper von *Drosophila melanogaster* wird durch geschlechtsspezifische Protein-Isoformen des Gens *doublesex* (*dsx*) gesteuert. In Weibchen bindet die weibliche Form des Proteins, DSX^F, an den Enhancer der Dotterproteingene und verstärkt die basale Trankriptionsrate. In Männchen wird die Transkription durch DSX^M vollständig unterdrückt. In der Stubenfliege *Musca domestica* dagegen scheinen Unterschiede in der Konzentration der Ecdysteroid-Hormone für die Regulierung der Dotterproteinsynthese verantwortlich zu sein. Die Dotterprotein-Konzentration in der Hämolymphe von Weibchen ist zyklisch, und der Ecdysteroidspiegel folgt demselben Zyklus. In Männchen, die keine Dotterproteine synthetisieren, bleibt die Ecdysteroidkonzentration auf konstant niedrigem Niveau. Durch die Injektion von 20-Hydroxy-Ecdyson kann man die Produktion von Dotterproteinen in Männchen induzieren. Es gibt allerdings Hinweise, dass weitere Faktoren zur Steuerung der Dotterproteinsynthese in Musca beitragen. Männchen reagieren beispielsweise auf die Injektion von 20E weniger stark als Weibchen. Weibchen, denen man die Ovarien entfernt hat, weisen nur noch eine niedrige Ecdysteroidkonzentration auf, vergleichbar mit jener in Männchen, aber sie produzieren trotzdem weiter Dotterproteine.

In meiner Dissertation habe ich untersucht, ob Transkriptionsfaktoren – neben Hormonen – in Musca an der Steuerung der Dotterproteinsynthese beteiligt sind. Für diese Untersuchung verwendete ich zwei Musca-Stämme, in denen Männchen kleine Mengen von Dotterproteinen produzieren. Die Tatsache, dass diese Männchen keine erhöhte Edysteroidkonzentration aufweisen, unterstützt die Hypothese, dass weitere Faktoren die Synthese von Dotterproteinen in Musca beeinflussen. Tatsächlich fanden wir in Musca ein Homolog von *dsx*, *Md-dsx*. *Md-dsx* wird, wie *dsx* in Drosophila, geschlechtsspezifisch gespleisst, und es entstehen zwei verschiedene Proteine, das weiblich-spezifische $Md\text{-}DSX^F$ sowie das männlich-spezifische $Md\text{-}DSX^M$.

Unsere Aufmerksamkeit galt sodann der Regulation von *Md-dsx*. Wir fanden dsx-repeat-elements (dsxRE) in *Md-dsx*, wie sie auch in den *dsx*-Homologen von *Drosophila melanogaster*, *Megaselia scalaris* und *Bactrocera tryoni* vorkommen. In Drosophila bindet der TRA/TRA2-Proteinkomplex an diese Elemente, wodurch die *dsx*-prä-mRNA zur weiblichen Form dsx^F gespleisst wird. Wir nahmen daher an, dass in Musca zu *tra* und *tra2* homologe Gene existieren, und es gelang uns, in Musca ein *tra2* Homolog zu identifizieren. Wir konnten zeigen, dass $Md\text{-}dsx^F$-Transkripte nur in Anwesenheit von Md-TRA2 hergestellt werden können, und dass *Md-tra2* für die weibliche Entwicklung essentiell ist. Vor kurzem konnten wir wir ausserdem ein Gen identifizieren, welches möglicherweise ein Homolog von *tra* ist.

In Standardmännchen ist nur $Md\text{-}dsx^M$, das männliche Transkript von *Md-dsx*, nach-

weisbar. In Männchen, die Dotterproteine produzieren, findet man dagegen auch *Md-dsx*^{F}. Hinzu kommt, dass durch ektopische Expression von Md-DSX^F in Standardmännchen die Synthese von Dotterproteinen induziert werden kann. Aufgrund dieser Resultate vermuteten wir, dass die Dotterproteinsynthese in Musca durch ein Zusammenspiel von Ecdysteroiden und DSX-Proteinen gesteuert wird. Wir haben folgendes Modell entwickelt: Im Fettkörper von Weibchen erhöht die Anwesenheit von Md-DSX^F die Kompetenz der Dotterproteingene, auf Ecdysteroide mit verstärkter Transkription zu reagieren. In Männchen dagegen verringert Md-DSX^M die Empfindlichkeit der Dotterproteingene und setzt die Schwelle für eine Aktivierung durch Ecdysteroide massiv hinauf. Hormone erfüllen zwei verschiedene Aufgaben in der Steuerung der Dotterproteinsynthese. Sie sind erstens verantwortlich dafür, dass die Dotterproteinsythese synchron mit der Oogenese verläuft. Zweitens ermöglichen Hormone die Anpassung der Dotterproteinproduktion an äussere Einflüsse, wie zum Beispiel an das Nahrungsangebot oder an das Vorhandensein von geeigneten Eiablageplätzen.

Es ist sehr wohl möglich, dass dieser Mechanismus – zellautonome Faktoren für die geschlechts- und gewebespezifische Expression, Hormone für die Anpassung an Umwelteinflüsse – auch für die Steuerung der Dotterproteinsynthese in anderen Insektenarten verwendet wird. Allerdings dürfte der Beitrag dieser beiden Systeme unterschiedlich sein, und zwar abhängig davon, wie die Entwicklung der Oocyten verläuft. Ist die Oogenese zyklisch, wie etwa in Musca, spielen Hormone eine viel wichtigere Rolle als in Drosophila, wo die Ei-Entwicklung kontinuierlich erfolgt, weil in Spezies mit zyklischer Oogenese die Produktion der Dotterproteine mit der Entwicklung der Eier synchronisiert werden muss.

3 Introduction

3.1 Regulation of yolk protein synthesis in insects

Yolk proteins (YP) are expressed in the female fat body of most insect species (HAGEDORN and KUNKEL, 1979); in some species, the follicle cells enveloping the growing oocyte also contribute to the production (BRENNAN *et al.*, 1981; JOWETT and POSTLETHWAIT, 1980). The YP are taken up by the oocyte and later used for the nutrition of the developing embryo (BOWNES, 1989; DEBIANCHI *et al.*, 1985).

Insect yolk proteins seem to be related to two different families of genes. The yolk protein genes of Drosophila show homology to vertebrate lipases (BOWNES *et al.*, 1988); the Ceratitis, Calliphora and Musca YP genes are highly conserved compared to the Drosophila *yp* genes (RINA and SAVAKIS, 1991; MARTINEZ and BOWNES, 1994; WHITE and BOWNES, 1997), and thus are also related to vertebrate lipases. YP of lower insects are more closely resemble the vitellogenin (*Vg*) genes of Xenopus and chickens (reviewed in BYRNE *et al.*, 1989). The function of the *yp* and Vg genes in different insect species, however, is the same – to serve as nutrition for the growing embryo..

YP synthesis can be controlled cell-autonomously by transcription factors of the sex-determining cascade, such as *doublesex* (*dsx*), or non-autonomously by a sex-specific concentration of hormones, such as ecdysteroids (reviewed in BOWNES, 1989). Regulation of YP synthesis is a very well suited model to study the control of sex-differentiating genes, since YP can easily be detected. Also, the coding and regulatory sequences of the YP genes of several species are known. Other sexual dimorphisms, like the formation of external and internal genitalia, depend on a complex network of intersecting pathways, and it is thus much more difficult to explore how they are controlled by the sex-determining genes. In many insect species, YP synthesis is regulated by hormones, in particular by juvenile hormone (JH) and ecdysteroids (IZUMI *et al.*, 1994).

In the migratory locust (*Locusta migratoria*, Orthoptera), vitellogenin (Vg) expression seems to depend mainly on JH (GLINKA *et al.*, 1995; DHADIALLA *et al.*, 1987; CHINZEI *et al.*, 1982), though there is some evidence that 20-hydroxy-ecdysone (20E) is also involved (GIRARDIE and GIRARDIE, 1996; GIRARDIE *et al.*, 1992; 1996; 1998). Vg expression in Locusta is sex- and stage-specific and correlates with the JH concentration in the hemolymph (GLINKA *et al.*, 1995). Application of JH can induce Vg synthesis in fourth and fifth instar larvae of both sexes and in adult females; interestingly, only trace amounts of Vg can be found in adult males after application of JH (DHADIALLA and WYATT, 1983). Therefore, additional factors must be present in adult females that enhance expression of the *Vg* genes.

The german cockroach, *Blattella germanica* (Blattaria), is another insect species where the production of vitellogenin is probably controlled by JH, since the levels of JH and yolk

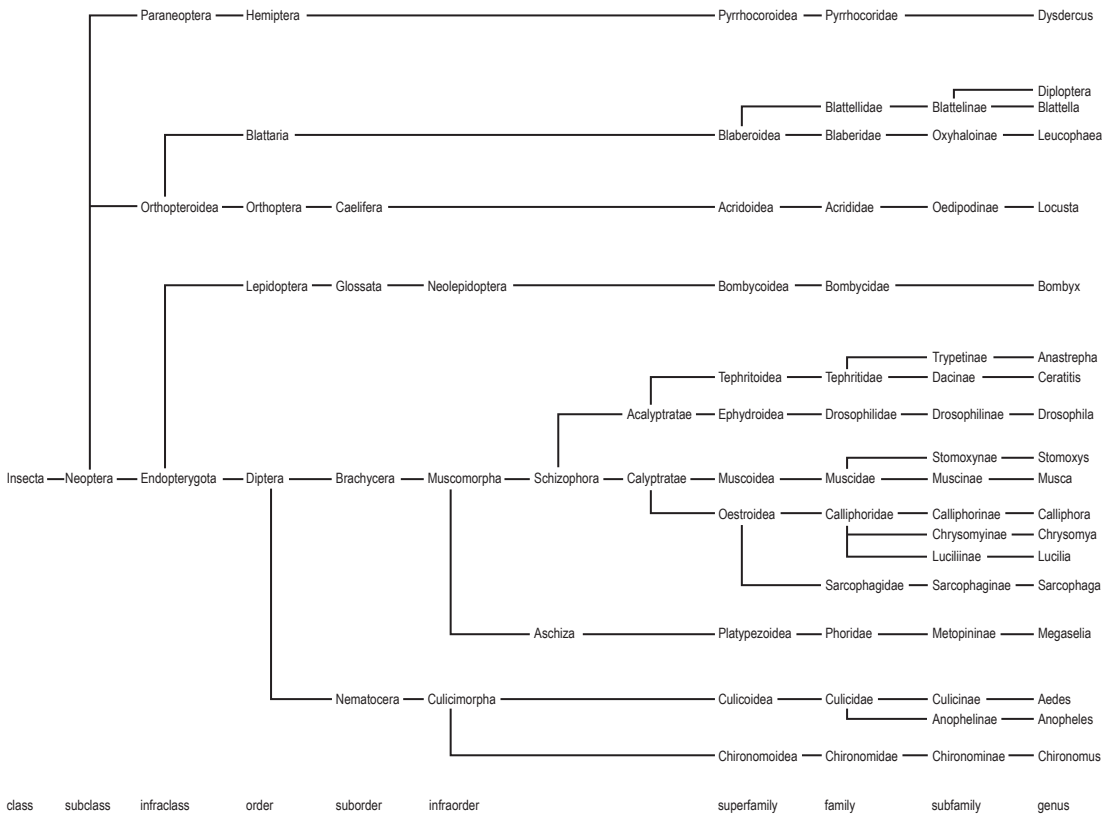

Figure 1: Overview over some insect species and their evolutionary relationship

protein in the hemolymph are correlated (CRUZ *et al.*, 2003).

In the red cotton stainer *Dysdercus koenigii* (Hemiptera), JH has two separate roles: Both vitellogenin synthesis and uptake of hemolymph vitellogenin into the oocyte depend on JH, whereas 20-hydroxy-ecdysone plays no apparent role (VENUGOPAL and KUMAR, 2000).

Other species use mainly 20E to control yolk protein synthesis, as is the case in females of the yellow fever mosquito *Aëdes aegypti*: A blood meal causes a burst of ecdysteroids, which will in turn trigger the expression of the vitellogenin genes by binding of the ecdysteroid receptor with its ligand 20E to regulatory sequences upstream of the *Vg* gene (DHADIALLA and RAIKHEL, 1994; MARTIN *et al.*, 2001). Other factors, like the transcription factor β-FTZ-F1 and juvenile hormone, seem to be necessary co-factors for *Vg* expression (ZHU *et al.*, 2003).

Injection of 20E is known to induce YP synthesis in males of several dipteran species, for example in Drosophila (KOZMA and BOWNES, 1986), *Musca domestica* (ADAMS *et al.*, 1989), Sarcophaga, Calliphora, Phormia and Lucilia (HUYBRECHTS and DE LOOF, 1982).

Arthropod species other than insects also rely on ecdysteroids for controlling YP syn-

thesis. In the spider *Tegenaria atrica*, mating causes an increase in the titer of 20E, which in turn induces the production of vitellogenin. In the tick Ixodes scapularis, Vg and ecdysteroid concentration in the hemolymph of females show a positive correlation; a peak of ecdysteroids six days after attachment to the host precedes an increasing rate of Vg synthesis (JAMES *et al.*, 1997).

In some orthopteran insects, ecdysteroids act as an inhibitor of vitellogenesis, for instance in the cockroaches *Leucophaea maderae* (ENGELMANN, 1971) and *Diploptera punctata* (FRIEDEL *et al.*, 1980).

Some insects are known where hormones do not play an apparent role in Vg synthesis; this is for example the case in the caribbean fruitfly, *Anastrepha suspensa*, or the stable fly, *Stomoxys calcitrans*. In both species, vitellogenins are exclusively synthesized by the ovaries (HANDLER 1997; HOUSEMAN and MORRISON, 1986; CHEN *et al.*, 1987). Therefore, no sex-specific regulation by hormones is necessary.

In contrast, the three *yp* genes in the fat body of Drosophila seem to be controlled directly by *dsx*, a transcription factor of the sex-determining cascade (COSCHIGANO and WENSINK, 1993). YP expression in the ovary, on the other hand, does not directly depend on genes of the sex-determining cascade. Instead, it is the formation of the ovaries that is controlled by the sex determining genes, and YP synthesis is a tissue-specific feature of an ovary (BOWNES *et al.*, 1990). In dsx^{-} mutants, the *yp* genes are expressed at a basal level, indicating that in females, the female protein form of *dsx* (DSX^{F}) enhances this weak expression whereas in males, the basal transcription is completely repressed by the male splice variant of *dsx* (DSX^{M}) (reviewed in BOWNES, 1994). Ecdysteroids do not seem to play a role in YP expression in Drosophila, since its titer is low in both females and males (BOWNES *et al.*, 1984). Nevertheless, YP synthesis can be induced in males by injection of 20-hydroxy-ecdysone (BOWNES *et al.*, 1983; SHIRK *et al.*, 1983).

All three YP genes (*yp1*, *yp2* and *yp3*) have been mapped to the X chromosome in Drosophila (BARNETT *et al.*, 1980). *yp1* and *yp2* are divergently transcribed and share a common enhancer of about 1.3kb, whereas *yp3* is located at a distance of several thousand kb (HUNG and WENSINK, 1983; Fig. 2). A 126bp sequence called fat body enhancer (FBE) in the intergenic spacer between *yp1* and *yp2* is sufficient to drive sex- and fat body-specific expression of *yp1* and *yp2* (SHEPHERD *et al.*, 1985; GARABEDIAN *et al.*, 1986). This FBE contains several binding sites for different proteins. Three *dsx* binding sites (dsxA, dsxB, dsxC) confer sex-specificity; a single dsxA site is already sufficient to drive sex-specific expression of a reporter gene, but it has no effect on tissue-specificity. Tissue-specificity is brought by three bzip motifs, overlapping with the dsxA site, to which the transcription factor DmC/EBP will bind. Two other binding sites could be identified: an aef1 motif, which seems to have no effect on either sex- or tissue-specificity, and ref1, a

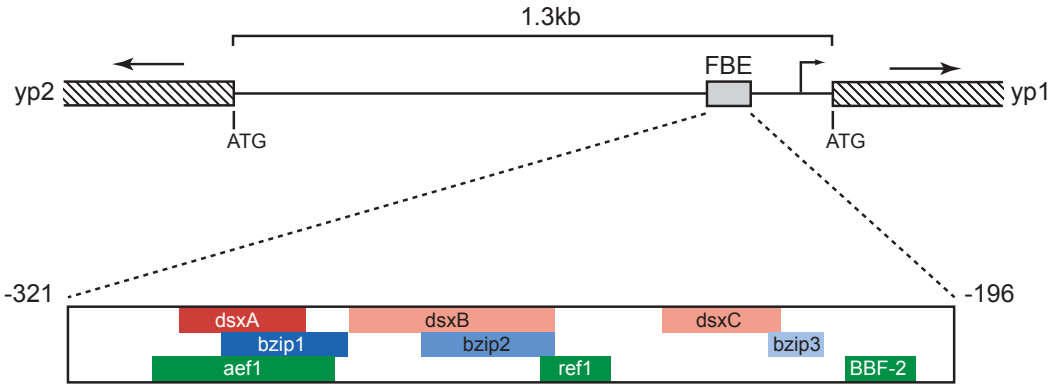

Figure 2: Organization of *yp1* and *yp2* of Drosophila (HUNG and WENSINK, 1983; AN and WENSINK, 1995; BURTIS *et al.*, 1991

binding site for an unknown positive regulator which activates transcription synergistically together with the bzip1 site (AN and WENSINK, 1995). The dsxA, dsxB and dsxC sites have been shown to bind DSX^F and DSX^M in vitro (BURTIS *et al.*, 1991).

In *Musca domestica*, females exhibit an ecdysteroid level that cycles in parallel to the YP concentration, and injection of 20-hydroxy-ecdysone into males induces transient YP synthesis (ADAMS and FILIPI, 1983; ADAMS *et al.*, 1985; AGUI *et al.*, 1985; ADAMS *et al.*, 1989). Nevertheless, there are indications that other factors may be involved: Males do not respond as well to injection of 20E as females; they are about 100 times less sensitive, and it takes twice as long until YP synthesis reaches its maximum. Moreover, ovaryectomized females continue to express YP, though their ecdysteroid titer drops to a very low, male-like level (AGUI *et al.*, 1991). Homologues of all three Drosophila *yp* genes have been identified in Musca. As in Drosophila, they are expressed in the female fat body and in the ovaries. mRNA abundance of all three YP was shown to correlate with ecdysteroid levels in females (WHITE and BOWNES, 1997). On the other hand, the enhancer region of *Mdyp1* contains several putative *dsx* binding sites, which are able to bind Drosophila DSX in vitro. Yet, a reporter construct containing 1 kb of this enhancer region showed tissue- but not sex-specific regulation in Drosophila: It was expressed in the ovaries as well as in female and male fat body (TORTIGLIONE and BOWNES, 1997). No ecdysteroid responsive elements have been identified in this enhancer region so far.

If we compare what is known about the regulation of YP synthesis in Musca and Drosophila, we can find similarities: Though *dsx* transcription factors play an important role in Drosophila, there are some indications that ecdysteroids might also be involved, since YP expression can be artificially induced in males by injection of 20E – but it is not known whether ecdysteroids take part in regulating YP in females. In Musca, we find the opposite situation: Ecdysteroids apparently are the main controlling agents in YP

synthesis, but male and female fat bodies respond differently, suggesting that sex-specific factors may also play a role. This contribution to YP regulation has not yet been analyzed.

In the lepidopteran species *Bombyx mori*, Bm-DSX proteins can bind to the enhancer region of the yolk protein genes in vitro, and ectopic expression of the female protein variant Bm-DSX^F causes males to produce small amounts of YP (SUZUKI *et al.*, 2003). We can summarize that in all species observed, YP production occurs exclusively in females, and that this strict sex-specificity can be achieved by three different mechanisms:

1. YP are expressed in female-specific tissues only, that is, in the ovary (e.g. Anastrepha, Stomoxys)

2. YP are regulated by a sex-specific hormonal milieu, either by ecdysteroids or juvenile hormone (e.g. Locusta, Aëdes, Musca, Blatella)

3. YP are controlled directly by sex-specific transcription factors, (e.g. Drosophila and, possibly, Bombyx).

All these mechanisms rely on the instructions relayed by the sex determination cascade. We chose to analyze YP synthesis in *Musca domestica*, in particular whether transcription factors like *dsx* also contribute to its regulation.

3.2 Sex determination in insects

The determination of which sexual fate an individual will adopt is a key event in the development of all higher eukaryotes. It starts with a primary sex-determining signal, which is relayed through a cascade of genes to a terminal regulator gene that activates or represses effector genes responsible for the formation of the various sexually dimorphic features. Thus, we can distinguish three steps that establish the sexual identity: Instruction (the primary signal), transduction (the cascade) and, finally, execution (the expression of sex-differentiating genes).

Primary signals are very diverse in nature, even within the minor taxonomic group of dipteran insects (MARIN and BAKER, 1998; SCHÜTT AND NÖTHIGER, 2000). The most common and ancestral mechanism seems to be the use of a dominant male determining factor (MARIN and BAKER, 1998), located either on a heteromorphic Y chromosome or on one of the homomorphic autosomes; it can be found in many dipteran species, such as Aëdes, Anopheles, Calliphora, Ceratitis, Chironomus, Megaselia and Musca. The chromosomal balance system of Drosophila, using a complex mechanism of X chromosome and autosome counting, seems to be a rather uncommon sex determining system (CLINE and MEYER, 1996). Dominant female determiners are found in certain populations of Musca (RUBINI, 1967; DÜBENDORFER *et al.*, 1992) and in the silkworm *Bombyx mori* (HASHIMOTO 1933). In *Chrysomya rufifacies*, sex of the offspring is determined by the

genotype of the mother; some females produce only sons (arrhenogenic females), others only daughters (thelygenic females; ULLERICH, 1984); sex determination by maternal genotype also exists in certain Musca strains (VANOSSI and ROVATI, 1982). Even environmental sex determination can be found in some dipteran species; in the sub-arctic mosquito *Aëdes stimulans*, e.g., sex of the offspring depends on the nutritional status of the mother (NÖTHIGER and STEINMANN-ZWICKY, 1985).

3.3 Sex determination in Drosophila and Musca

Since sex determination of *Drosophila melanogaster* is best known among insects, we used this system as a reference for studies in *Musca domestica*. In Drosophila (Fig. 3), the primary signal is the ratio of the number of X chromosomes to sets of autosomes, the so-called X:A ratio (for a review, see for instance SCHÜTT and NÖTHIGER, 2000). Males (XY) have only one X chromosome and therefore a ratio of 1:2 = 0.5, females (XX) have two X chromosomes and a X:A ratio of 2:2 = 1. The Y chromosome of the males does not play a role in sex determination. It contains very few genes, but some of them are necessary for sperm motility. X0 males are sterile. In contrast, the Drosophila X chromosome contains many genes; hence, animals without an X chromosome are not viable.

In females, an X:A ratio of 1 activates the early promoter of the *Sex-lethal* (*Sxl*) gene; in males, *Sxl* is not activated in the early embryo. The SXL protein, a splice factor, acts upon its own pre-mRNA such that in females, exon 3 of the *Sxl* pre-mRNA will be spliced out, thus removing stop codons. In males, where no early SXL protein is made since the early promoter is not activated, exon 3 with its stop codons will be included in the mature mRNA, and a full-length SXL protein cannot be produced. Thus, *Sxl* regulates its own activity in females through an autoregulatory loop. The SXL splice factor also acts upon the next gene in the cascade, *transformer* (*tra*). In females, a functional TRA protein is made, since the presence of SXL protein allows splicing out of the first part of exon 2, which contains stop codons; in males, where this sequence cannot be spliced out, these stop codons lead to premature termination of the translation.

One terminal regulator of the Drosophila sex-determining cascade is the transcription factor *dsx*. TRA/TRA2 protein dimers, made of female TRA and the non-sex-specific co-factor TRA2, activate a weak 3' splice acceptor site at the beginning of the female exon 4 in the *dsx* gene, and consequently, the female DSX^F protein is produced. In males, splicing together exons 1, 2, 3 and 5 gives rise to the default male splice variant DSX^M. Both forms of *doublesex* proteins contain a DNA binding domain forming an atypical zinc finger, which is encoded in the common exon 2, but they differ at the carboxyterminal end (BAKER *et al.*, 1989; ERDMAN and BURTIS, 1993), where an oligomerization domain can be

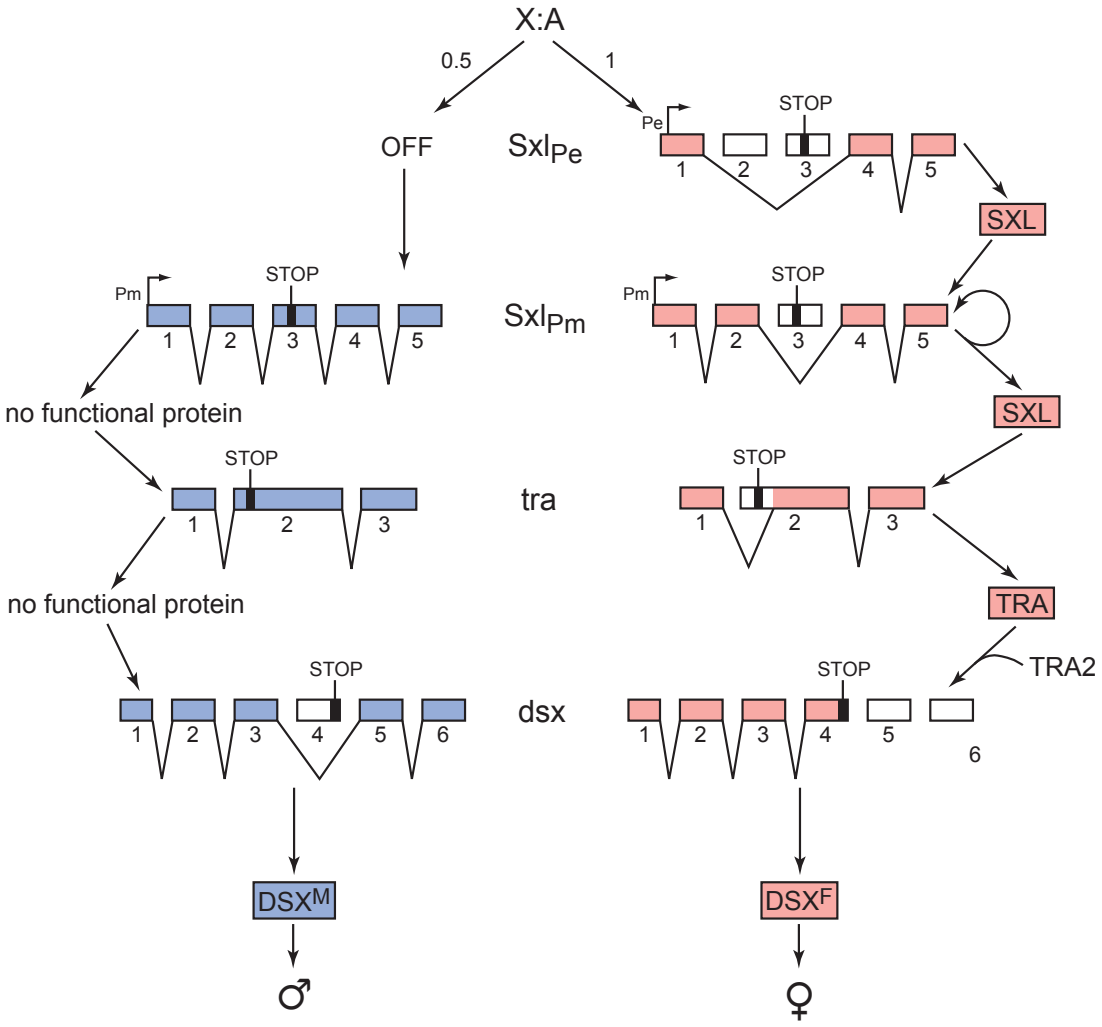

Figure 3: Sex determination in *Drosophila melanogaster*

found in females. Thus, both protein isoforms will bind to the same DNA sequences, but could interact with different proteins, and consequently exert different functions in males and females. The female form of the protein, DSX^{F}, enhances female- and represses male-differentiating genes; the male-specific DSX^{M} acts in the opposite direction. The *dsx* transcription factor is responsible for sexual dimorphisms of the external and internal morphology, such as the genitalia, and the sex-specific expression of yolk proteins (reviewed in BAKER *et al.*, 1989; SLEE and BOWNES, 1990).

In Musca, four different systems of sex determination are known (reviewed in DÜ-BENDORFER *et al.*, 2002). Most often, we find heteromorphic sex chromosomes: Males are, as in Drosophila, normally XY, females have the genotype XX. Both the X and the Y chromosomes of Musca consist mostly of heterochromatin and seem to be genetically equivalent; presence of one of these sex chromosomes is sufficient for survival. X0 animals are fertile females, whereas Y0 animals are fertile males. The primary signal is a male-

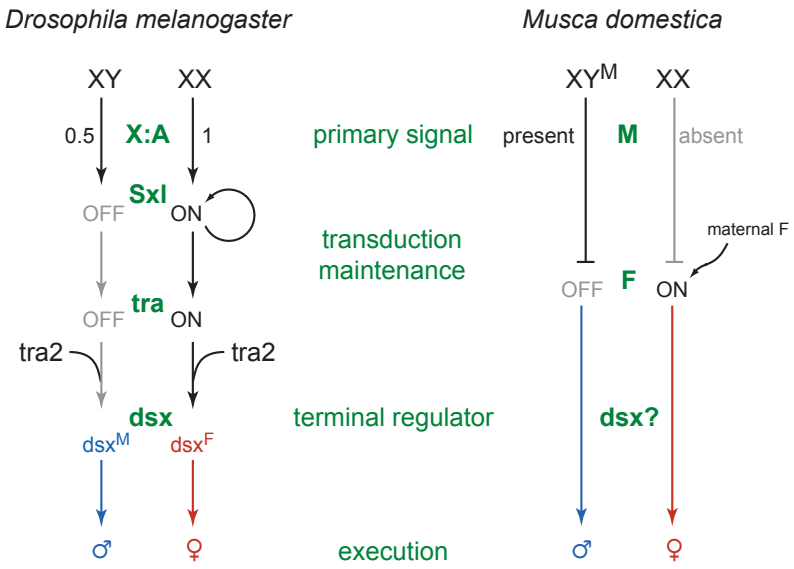

Figure 4: Comparison of the sex-determining systems of *Musca domestica* and *Drosophila melanogaster*

determining factor (*M* or *M* factor) on the Y chromosome.

The Musca homologue of the Drosophila *Sxl* gene has been identified some years ago, but its function is still unknown. *Md-Sxl* does not seem to play a role in sex determination, since the same transcripts and protein variants are present in males and females (MEISE *et al.*, 1998). Instead, the presence of the *M* factor on the Y chromosome represses a female-determining gene *F* on chromosome 4, which is genetically characterized, but whose molecular structure and function are not yet known. In females, absence of an *M* factor, together with maternally provided *F* protein or *F* mRNA, leads to activation of the zygotic *F* gene and subsequently to the female-specific splicing of the *Md-dsx* transcript and the development of a female (Fig. 4).

3.4 Variations of the sex determination system in Musca

Musca domestica is an extremely interesting object to study sex determination mechanisms, since nearly all the systems found in other dipterans are present within this one species: Dominant male determiners (on a heteromorphic Y chromosome or autosomal), a dominant female determining factor and a recessive male determiner (reviewed in DÜBENDORFER *et al.*, 2002; Fig. 5). All these variations are now understood as derivations of the standard sex-determining cascade of Musca, arisen by mutations in the *M* and *F* genes. Variations of the Musca sex determining systems can be found on the level of *M* as well as on the level of *F*.

In standard strains, the *M* factor is located on the Y chromosome; in these strains,

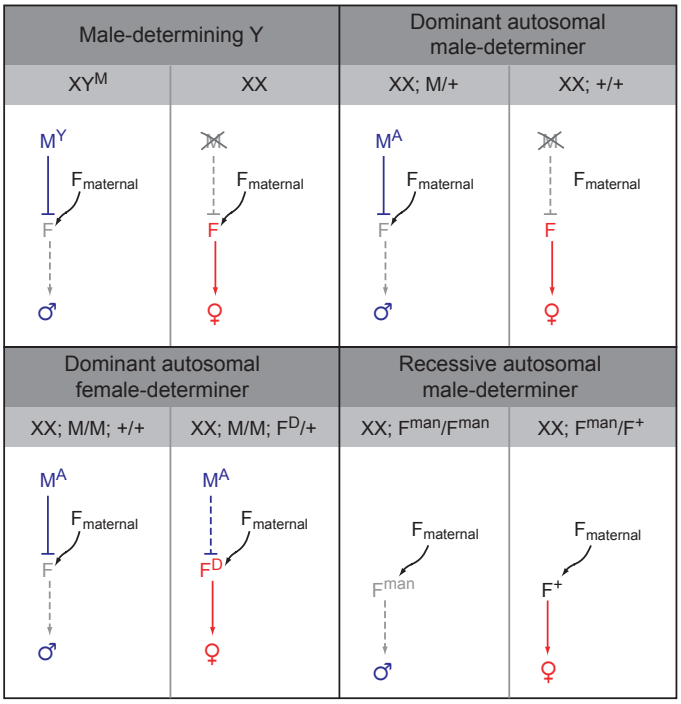

Figure 5: The different sex determination strategies of *Musca domestica*

the primary signal is thus a male-determining Y chromosome. One variation of Musca sex determination can be found in strains where M is located on one of the five autosomes or on the X chromosome. In these strains, there are no sex chromosomes; males and females both have the genotype XX, and sex is determined autosomally. The M factors on the different chromosomes are equivalent, though it is not clear whether they are molecularly identical. It has been speculated that M might be a mobile element. Some of these M factors, though, appear to be not fully functional, perhaps because they inserted near heterochromatic regions (SCHMIDT *et al.*, 1997b). For example, the M factor on chromosome I allows these otherwise normal and fertile M^I males to produce small amounts of yolk proteins (SCHMIDT *et al.*, 1997b). Other indications that M^I may be slightly different were found when male pole cells were transplanted into females. Eggs derived from transplanted pole cells containing an M^Y give rise to exclusively male offspring, even if they are XX, since M^Y in the germ line represses the maternal F, and zygotes without maternal F product cannot develop as females (HILFIKER-KLEINER *et al.*, 1994). Eggs derived from transplanted pole cells of the genotypeX/M^I do not only give rise to males, but also to females and intersexes, indicating that M^I is not able to completely repress the F gene in the maternal germline (SCHMIDT *et al.*, 1997b). We thus considered M^I to be a 'weak' M.

Two variations of the Musca sex determination cascade have arisen by mutations in the F gene. One allele of F seems to be a gain-of-function mutation. This F^D ($F^{Dominant}$)

is epistatic over M: Every animal carrying one copy of F^D develops as a female, even in the presence of one or several M factors. In such strains, animals are usually homozygous for an autosomal M, and there is no Y chromosome (RUBINI, 1967). The other known F mutation has arisen in our lab and is called F^{man} ($F^{masculinizer}$); it is a hypomorphic F allele. Animals homozygous for F^{man} are males, but like M^I males, they synthesize small amounts of yolk proteins (SCHMIDT *et al.*, 1997a); additionally, F^{man} males exhibit a somewhat reduced fertility due to behavioral defects (S. Käppeli, unpublished results). Animals of the F^{man} strain carry neither a Y chromosome nor an autosomal M factor. This sex-determining system is thus based on function or non-function of F, without controlling M.

3.5 *Sxl* and *dsx* in different insect species

Sxl homologues have been identified in several other dipteran species, namely *Ceratitis capitata* (SACCONE *et al.*, 1998), *Chrysomya rufifacies* (MULLER-HOLTKAMP, 1995), *Megaselia scalaris* (SIEVERT *et al.*, 1997; Sievert *et al.*, 2000) and *Anopheles gambiae* (PANNUTI *et al.*, 2000). Unlike in Drosophila, *Sxl* is not sex-specifically expressed in any of these species. Additionally, the Musca and Ceratitis *Sxl* genes have no sex-transforming effect when expressed in Drosophila (MEISE *et al.*, 1998; SACCONE *et al.*, 1998). Thus, it seems that *Sxl* is a relatively recently recruited member of the sex-determining cascade in Drosophila.

In contrast, *dsx* homologues found in Megaselia (SIEVERT *et al.*, 1997; Kuhn *et al.*, 2000), Anopheles (PANNUTI *et al.*, 2000), Ceratitis (Saccone *et al.*, 1996), *Bactrocera tryoni* (SHEARMAN and FROMMER, 1996) and the silkworm *Bombyx mori* (OHBAYASHI *et al.*, 2000) all show a sex-specific splice pattern. These findings - conservation of the terminal regulator *dsx*, but not of the upstream regulatory element *Sxl* - support a model proposed by Wilkins (1995), postulating that sex-determining pathways have evolved bottom-up, i.e. the most ancestral and most conserved genes would be found at the bottom of the cascade, whereas those at the top would be more recent recruitments. Since *dsx* is the conserved terminal regulator of the sex determination cascade in many insect species, it seems very likely that a homologue will also be present in *Musca domestica*.

In my thesis work, I addressed two questions:

1. Does a *dsx* homologue exist in Musca, and what would be its position in the sex determination cascade?

2. Does *Md-dsx* contribute to the regulation of yolk protein synthesis?

4 Papers

4.1 Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*.

Hediger, M., Burghardt, G., Siegenthaler, C., Buser, N., Hilfiker-Kleiner, D., Dübendorfer, A., Bopp, D. (2004)

Dev Genes Evol 214 (1): 29-42

In this work, we report the cloning and characterization of the housefly *doublesex* homologue, *Md-dsx*. We analyzed the consequence of ectopic expression of the male- and female-specific splice variants of *Md-dsx*, $Md\text{-}dsx^M$ and $Md\text{-}dsx^F$, on morphology and physiology of transgenic animals. I contributed to this work by analyzing the expression of the Musca yolk protein genes in transgenic animals, showing that expression of $Md\text{-}dsx^F$ in males is sufficient to induce YP synthesis, a typical female physiological response.

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ORIGINAL ARTICLE

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Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*

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Abstract Sex-determining cascades are supposed to have evolved in a retrograde manner from bottom to top. Wilkins' 1995 hypothesis finds support from our comparative studies in *Drosophila melanogaster* and *Musca domestica*, two dipteran species that separated some 120 million years ago. The sex-determining cascades in these flies differ at the level of the primary sex-determining signal and their targets, *Sxl* in *Drosophila* and *F* in *Musca*. Here we present evidence that they converge at the level of the terminal regulator, *doublesex* (*dsx*), which conveys the selected sexual fate to the differentiation genes. The *dsx* homologue in *Musca*, *Md-dsx*, encodes male-specific ($MdDSX^M$) and female-specific ($MdDSX^F$) protein variants which correspond in structure to those in *Drosophila*. Sex-specific regulation of *Md-dsx* is controlled by the switch gene *F* via a splicing mechanism that is similar but in some relevant aspects different from that in *Drosophila.* $MdDSX^F$ expression can activate the vitellogenin genes in *Drosophila* and *Musca* males, and $MdDSX^M$ expression in *Drosophila* females can cause male-like pigmentation of posterior tergites, suggesting that these *Musca dsx* variants are conserved not only in structure but also in function. Furthermore, downregulation of *Md-dsx* activity in *Musca* by injecting dsRNA into embryos leads to intersexual differentiation of the gonads. These results strongly support a role of *Md-dsx* as the final regulatory gene in the sex-determining hierarchy of the housefly.

Keywords *Musca domestica* · Sex determination · *doublesex* · Alternative splicing

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Introduction

Insects employ a variety of seemingly different strategies to determine sex (Nothiger and Steinmann-Zwicky 1985). This variability becomes evident even in one single species, the housefly *Musca domestica*, where different modes of sex determination have been found in naturally occurring populations (Milani 1967). These observations suggested the presence of a short genetic cascade for the control of sexual development in *Musca*: a dominant male-determining factor, *M*, represses the key gene for sex determination, *F*, which leads to male development. Absence of *M* and presence of maternal *F* product in the zygote are the prerequisites for *F* activity, which results in female development (for review see Dubendorfer et al. 2002).

In most wild strains, *M* is located on the Y chromosome, but strains in which both sexes are XX and males carry an autosomal *M* in heterozygous condition also exist (Rubini et al. 1972). Other wild populations are known where both sexes are homozygous for *M*, and females are heterozygous for the dominant gain-of-function allele F^D which overrules the male-determining function of *M* (Milani 1967). Yet another type of sex-determining system operates without *M*. In this strain, which arose in our laboratory, males are homozygous for a putative recessive loss-of-function mutation of *F*, F^{man}, whereas females are heterozygous for the mutation (Schmidt et al. 1997). Finally, Vanossi Este and Rovati (1982) described a system of maternally controlled sex determination, where *Ag*/+ females, due to a failure to activate *F* in the germline (Hilfiker-Kleiner et al. 1993), are arrhenogenic and produce sons, whereas +/+ females are thelygenic and, with males of this strain, produce exclusively daughters. We have proposed that all these different modes of sex determination in *M. domestica* are based on single mutations in an otherwise invariant set of genes, rather than on major alterations in the genetic architecture

of the pathway that controls sexual development (Dubendorfer et al. 2002).

To identify the regulatory components involved in *Musca* sex determination, we initiated a comparative analysis with the well-characterised cascade of *Drosophila melanogaster* as a reference. Our objective was to isolate genes in *Musca* based on homology to the sex-determining genes in *Drosophila* and to test them for a possible role in sexual differentiation. This type of analysis is expected to unveil the extent of correspondence between the two sex-determining pathways. Dissimilarities are found in the primary signal that determines the sexual fate of the embryo. *Drosophila* does not employ dominant male or female determiners, but rather the number of X-chromosomes relative to sets of autosomes, the X:A ratio, as a primary signal for sex determination (Cline 1993). Genetic studies in *Musca* revealed a binary switch gene, *F*, which is controlled by the primary signal *M*. The functional correspondence of Sex-lethal (*Sxl*) and *F* does not, however, extend to the molecular level. In a previous study, we have demonstrated that the *Musca* homologue of *Sxl* is equally expressed in both sexes which makes it an unlikely candidate for *F* (Meise et al. 1998). The possibility remains that *F* corresponds to one of the more downstream genetic switches in the pathway of *Drosophila*.

In *Drosophila*, *Sxl* transduces the selected fate—female when on and male when off—through the switch gene *transformer* (*tra*) to the last gene in the cascade, *double sex* (*dsx*; reviewed in Baker et al. 1989). *dsx* encodes two sex-specific variants of a transcription factor that share an atypical zinc finger domain, but differ in their carboxy-terminal parts (Baker et al. 1989; Erdman and Burtis 1993). The male and female proteins control transcription of the terminal differentiation genes with opposite activities. The female-specific DSX protein, DSX^F, directs female development by promoting transcription of female-specific differentiation genes and repressing male-specific differentiation genes, while the male-specific DSX protein, DSX^M, acts in the opposite direction (reviewed in Baker et al. 1989; Slee and Bownes 1990). Sex-specific expression of *dsx* is achieved at the level of differential splicing and depends on the activity of *tra* and *transformer2* (*tra2*). In females, active TRA products, in combination with TRA2 proteins, bind to splice enhancer sites in the *dsx* pre-mRNA to promote the use of a weak female-specific 3′ acceptor site (Hedley and Maniatis 1991; Hertel et al. 1996; Ryner and Baker 1991; Tian and Maniatis 1993). This splice pattern leads to the inclusion of an exon that encodes the female-specific carboxy end (Fig. 1a). In males, where no active TRA is present, this site is not recognized, and two downstream exons are included that encode the male-specific carboxy end (Fig. 1a).

In this study, we have identified the *dsx* homologue in *Musca*, *Md-dsx.* Our results confirm a role of *Md-dsx* in sexual differentiation of the housefly. Sex-specific regulation of *Md-dsx* is achieved at the post-transcriptional level, but shows some interesting deviations from the mechanism that operates in *D. melanogaster.*

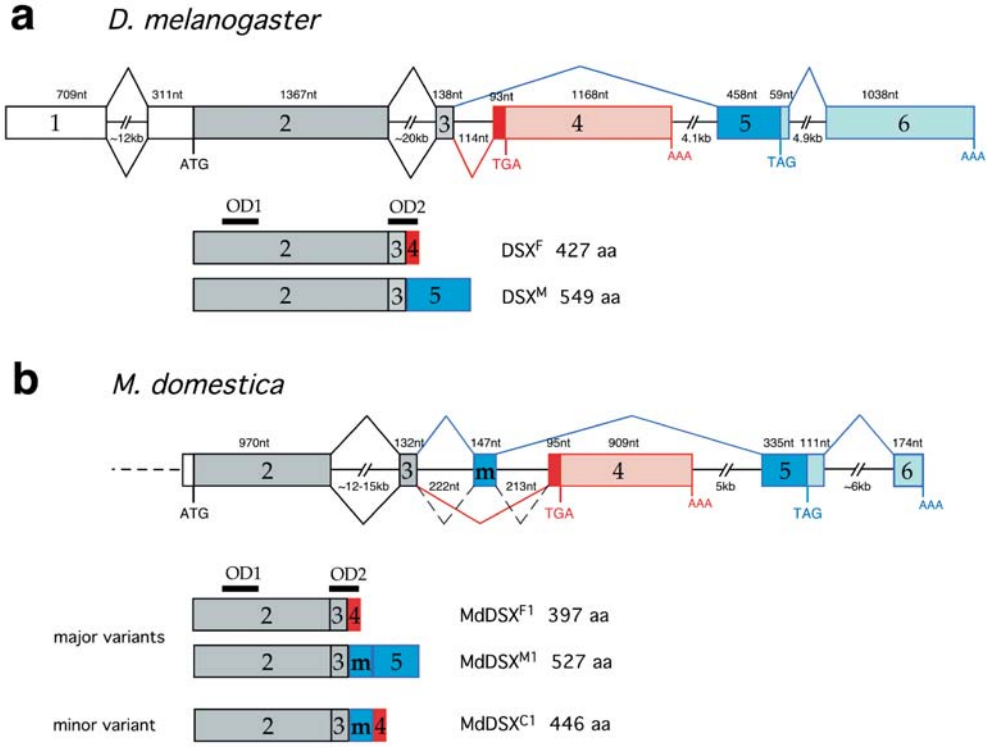

Fig. 1 Schematic drawing of the genomic organization and the structure of splice variants of *dsx* in *Drosophila* (**a**) and *Musca* (**b**). Male-specific exons are marked in *blue* and the female-specific exon in *red*. Note that exon m in the *Musca* gene has no correspondence in the *Drosophila* gene. Exon and intron sizes are indicated in nucleotides (nt), translational start and stop sites as well as the poly(A) addition sites are *marked*

Materials and methods

PCR with degenerated primers

The 5′ primers correspond to sequences located in exon 3, and 3′ primers in the female-specific exon 4 of the *dsx* gene of *D. melanogaster.* One pair of degenerated primers, DSXC and DSXF2, was kindly provided by Dr. Guiseppe Saccone (University of Naples, Italy) and another pair, FHC3 and FHC4, we obtained from Dr. Antonio Pannuti (Emory University, Atlanta, United States).

DSXC 5′	GAR AAR TTY MGY TAY CCI TGG
DSXF2 3′	DAT RTT IAR RTT RTG YTG IC
FHC2 5′	CTI (CT)TI GA(GA) AA(AG) TT(TC) (CA)GI TA(TC) CCI TGG
FHC4 3′	T(GT)(TC) TGI C(GT)I GA(AG) TA(TC) TC(AG) TTI ACI AC

Musca templates were prepared from male and female cDNA of our wild-type (XX; XY) strain. A first PCR round was performed with DSXC and DSXF2 primers followed by a second amplification with FHC2 and FHC4. We used standard concentrations of Mg^{++} and nucleotides (Expand Long Template PCR System, Boehringer Mannheim). In a total volume of 50 µl, 50 ng DNA template was amplified with 20 µM of each primer. The following conditions were used: denaturation at 95°C for 5 min with subsequent addition of Taq polymerase, then 35 cycles (denaturation 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min), and lastly extension at 72°C for 7 min. Subcloning and sequencing of the candidate fragments were carried out by standard procedures.

Rearing of *Musca* strains

Strains were reared as described previously (Schmidt et al. 1997). Since small populations of larvae are difficult to raise on standard medium, larvae obtained from injected embryos were raised on porcine faeces. To kill *Musca* eggs and larvae of natural populations, dung was frozen at –20°C for several days and thawed before supplementing to standard medium.

Strains of *M. domestica*

The strains were: (1) wild-type strain, females *XX; +/+*, males *XY; +/+*; (2) NoM strain, females *XX;* F^{man}/F^{+}, males *XX;* F^{man}/F^{man}; (3) F^{D} strain, females M^{III}/M^{III} ; F^{D}/F^{+}, males M^{III}/M^{III}; F^{+}/F^{+}; (4) *white* strain, females *XX; w/w*, males *XY; w/w*; (5) autosomal *M* strain, females *XX; pw bwb / pw bwb*, males *XX;* M^{III} pw^{+} bwb^{+}*/pw bwb*; (6) NoM strain, females *XX; +/+* (thelygenic) or *XX; Ag/+* (arrhenogenic), males *XX; +/+* or *XX; Ag/+* (Vanossi Este and Rovati 1982).

Crosses to obtain unisexual progenies were: female only (a) virgin females of strain 1 × males of strain 6, or (b) virgin females of strain 1 × males of strain 2; male only (c) virgin females of strain 1 × males of strain 3.

Northern blot analysis

Total RNA of about 200 mg flies [14 adult males (cross c) or 12 adult females (cross b)] was extracted with the AGPC-technique (Chomczynski and Sacchi 1987). Poly(A)$^{+}$ RNA was isolated using the Oligotex mRNA Maxi Kit of Qiagen. Poly(A)$^{+}$ RNA (10 µg per lane) was electrophoresed on a 1% agarose gel using the glyoxal technique. RNA was transferred by blotting with 10× SSPE to Hybond-N^{+} nylon membranes (Amersham). Filters were preincubated for 2 h at 42°C in hybridization buffer (50% formamide, 4× SSPE buffer, 5× Denhardt's solution, 1% SDS, 10 mM Tris-HCl pH 7.5, 0.1 mg/ml salmon sperm). Filters were then subsequently incubated overnight at 42°C in hybridization buffer containing 10^{6} cpm/ml radiolabeled RNA probes. RNA probes were generated by in vitro transcription of a cDNA template of the common part of *dsx* (*dsx*C) in the presence of [α-^{32}P]-CTP and T7 RNA polymerase. Filters were washed twice for 15 min in 2× SSPE at room temperature, then twice for 45 min in 2× SSPE/2% SDS at 65°C and finally two times in 0.1× SSPE for 15 min at room temperature. Filters were exposed overnight or longer on Fuji RX films.

RT-PCR analysis

The total RNA of three adult flies (around 50 mg) was extracted according to the RNeasy Mini protocol of Qiagen. RT-PCR reactions were performed using the Titan One Tube RT-PCR Kit (Roche) and *Md-dsx*-specific primers following the manufacturer's instructions.

Transgenic constructs and germline transformation

Drosophila

The *Drosophila* transformation vectors pUAST-*Md-dsx*F1 and pUAST-*Md-dsx*M1 were created by introducing a 1.4-kb *Eco*RI/*Xba*I cDNA fragment of *Md-dsx*F1 and a 1.8-kb cDNA fragment of *Md-dsx*M1 containing the whole open reading frames into the *Eco*RI/*Xba*I sites of the pUAST vector (Brand and Perrimon 1993).

The pHermes{Act5C-*Md-dsx*M1} construct was made by introducing the 1.8-kb *Md-dsx*M1 *Eco*RI/*Xba*I cDNA fragment into the *Bam*HI/*Xba*I sites of the pHAct5cEGFP construct (Pinkerton et al. 2000). The pHermes{Act5C-*Md-dsx*M1} construct was co-injected with the helper plasmid pKHSHH (500 ng/µl each) which expresses the Hermes transposase under the control of the *Drosophila* hsp70 promoter (Sarkar et al. 1997). As a host for germline integration *Drosophila* embryos of strain w^{1118} were used.

Musca

The pBac{3xP3-eGFP;hsp82-*Md-dsx*F1} transgene was constructed as follows: an hsp82 promoter/actin5C poly(A) signal fragment was isolated from pKhsp82 (Coates et al. 1996) and a 1.4-kb *Md-dsx*F1 *Eco*RI/*Xba*I cDNA fragment was placed between the hsp82 promoter and the actin5C poly(A) signal. This hsp82-*Md-dsx*F1-actin5C fragment was inserted into the FseI/AscI sites of the pBac{3xP3-eGFP}afm vector (Horn and Wimmer 2000). Twenty micrograms of pBac{3xP3-eGFP; hsp82-*Md-dsx*F1} vector was coprecipitated with 4 µg phsp-pBac helper plasmid containing the pBac-transposase under the control of the hsp70 promoter of *D. melanogaster* (Handler and Harrell 1999) and taken up in a volume of 30 µl injection buffer. Preblastoderm embryos of the *M. domestica* strain 4 were injected as described previously (Hediger et al. 2001).

Injection of dsRNA

cDNA fragments of the common (*dsx*C), female-specific (*dsx*F) and male-specific parts (*dsx*M) of the *Musca dsx* gene were produced by PCR flanked by T7 promoter sequences at their 3′ and 5′ ends. The *dsx*C fragment is part of exon 2 containing the entire OD1 and has a length of 480 bp. The *dsx*F fragment contains coding and noncoding parts of exon 4 and is 640 bp long. The *dsx*M fragment spans exons "m" and 5 and has a length of 560 bp. To produce dsRNA, the three cDNA fragments were transcribed in vitro using T7 RNA polymerase. The dsRNA was ethanol-precipitated and resuspended in injection buffer (final concentration 1 µg/µl).

Embryos were collected 0–1 h after egg laying (preblastoderm stage), dechorionated and injected as described by Hediger et al. (2001). Injected embryos were allowed to develop at room temperature.

Immunoblot analysis

Drosophila

Males carrying the pUAST-*Md-dsx*F1 construct together with the hsp70-GAL4 driver were treated with a twice-repeated heatshock pulse of 1 h at 37°C followed by 3 h at 25°C. Ten males and, for control, five females were then homogenized in 100 µl 2× SDS loading buffer. Samples were boiled for 5 min and insoluble material was removed by centrifugation. Of the supernatant 5 µl was loaded per lane and separated on 12% SDS-PAGE. After electrophoresis, protein was electrotransferred to a nitrocellulose membrane in Tris-glycin-methanol. Membranes were blocked in 4% low-fat dry milk powder in TBS/0.05% Tween-20 (TBST). We used a polyclonal anti-yolk protein antibody from *D. melanogaster* (gift from M. Bownes, University of Edinburgh) at a dilution of 1:5,000 in TBS/0.05% Tween-20/1 mg/ml BSA. For subsequent detection of the antigen-antibody complex, we used the alkaline phosphatase-conjugated anti-rabbit antibody (Promega) at a dilution of 1:7,500.

Musca

Animals carrying the pBac{hsp82-*Md-dsx*F1} construct were kept at 25°C and treated every 5 h with a 1 h heatshock pulse of 42°C from the early embryo stage until 5 days after eclosion. On the sixth day of adult life, 1 µl haemolymph was drawn from a single male with a fine glass needle. The haemolymph was mixed with 12 µl 2× SDS loading buffer and the samples were separated on 12% SDS-PAGE followed by transfer to a nitrocellulose membrane as described earlier. We used a polyclonal anti-yolk protein-antibody from *M. domestica* (kindly provided by Dr. T. Adams, Fargo, N.D.) at a dilution of 1:20,000 in TBS/0.05% Tween-20+1 mg/ml BSA. Detection of the antigen-antibody complex on the blot was done as described earlier.

Results

Isolation of the *dsx* homologue in *M. domestica*

Using a set of degenerated *dsx* primers, we amplified an 84 bp fragment from cDNAs prepared from total RNA of female *Musca* larvae (see Materials and methods). The sequence of this fragment, located between the DM domain (exon 3) and the female-specific domain (exon 4), displays a high degree of sequence similarity at the nucleotide level (68%) and at the amino acid level (82%) when compared to *dsx* sequences of *D. melanogaster.* With this fragment as a probe, two lambda clones (GEM11.14 and GEM11.18) were isolated from a genomic *Musca* DNA library (Tortiglione and Bownes 1997). An alignment of the phage insert sequences with the partial female cDNA sequence exposed an intron at exactly the same position as in *Drosophila* (Fig. 1a, b). This intron is 582 bp long and larger than the corresponding 114 bp intron in *Drosophila* between exons 3 and 4 (Baker et al. 1989). Interestingly, this intron in the *Musca* sequence harbours an additional exon that is preferentially included in transcripts isolated from males (see later). This additional exon has a length of 147 bp and was termed "m" for *Musca*- and male-specific.

To retrieve full-length cDNA sequences, we extended the cDNA fragment on both sides by 5′ and 3′ RACEs. Templates were synthesized from total RNA prepared from male and female third instar larvae. 5′ RACE on female templates led to the isolation of sequences corresponding to exon 2 of *Drosophila dsx;* and 3′ RACE on male cDNA templates revealed two exonic sequences downstream of exon 3 that are not co-linear with the previously isolated exon 4 sequences from female templates (Fig. 1b). We therefore suspected that these sequences are male-specific and termed them exon 5 and 6. Exon 2, 5 and 6 sequences are not contained within the genomic DNA inserts of GEM11.14 and GEM11.18 indicating that the intron between exons 2 and 3 must be larger than 14 kb, and the intron between exons 3 and 5 larger than 4 kb. In *Drosophila*, the intron between exons 2 and 3 has a size of about 23 kb, and the intron between exons 3 and 5 is 4.9 kb (Baker et al. 1989). Given the considerable similarity in structure and organization, we referred to this gene as *Md-dsx*.

Md-dsx encodes protein variants with structural similarity to DSX^M and DSX^F in *Drosophila*

We isolated a cDNA from male flies composed of exons 2-3-m-5-6 and termed this clone *Md-dsx*M1 (Fig. 1b). The major female variant composed of exons 2-3-4 was termed *Md-dsx*F1 (Fig. 1b). Both cDNAs represent two major splice variants of *Md-dsx* (Genbank AY461853 AY461854) and display a high degree of identity at the amino acid level to the corresponding variants in *Drosophila*. *Drosophila* DSX protein essentially consists of two domains, OD1 and OD2, which serve as interfaces for protein and DNA interactions (An et al. 1996; Cho and Wensink 1997). OD1 is composed of an atypical zinc-finger domain (DM) which directly binds to target sequences on the DNA. OD2 is an oligomerization domain that extends into the female-specific part of DSX^F. The longest ORF of *Md-dsx*F1 starts at an AUG in position 62 in exon 2 and stops 95 bp downstream of the acceptor site of exon 4, coding for a protein of 397 aa (Fig. 2). The predicted protein contains a conserved OD1 domain with only five non-conservative changes in a stretch of 63 residues (Fig. 2a). This high degree of similarity extends to the amino-terminal end of the protein upstream of OD1. Likewise, the region corresponding to OD2 is very similar in sequence. Here, only seven non-conservative changes are found in a stretch of 64 residues. In particular, the female-specific domain (32 aa) encoded by exon 4 is virtually identical in both species (Fig. 2b). On the other hand, the region that links OD1 and OD2 is poorly conserved and, like in *Drosophila*, of low complexity containing an unusually large number of histidine, glycine and alanine residues (Fig. 2a).

The ORF of the male transcript *Md-dsx*M1 extends from the same AUG in exon 2 to a translational stop in exon 5 which is located 335 bp downstream of the acceptor site. The predicted protein encoded by *Md-dsx*M1 is 527 aa. In contrast to the female-specific

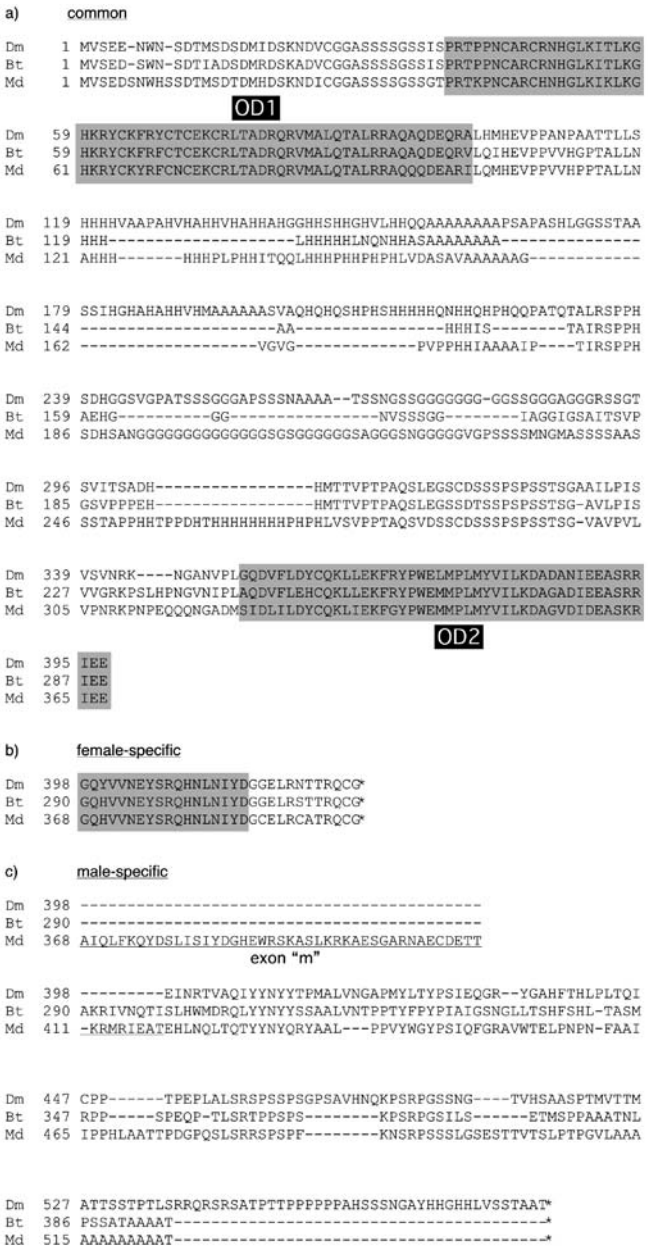

Fig. 2a–c Protein sequence alignment of *dsx* in *Drosophila melanogaster* (*Dm*; Burtis and Baker 1989), *Bactrocera tryoni*, (*Bt*; Shearman and Frommer 1998) and *Musca domestica* (*Md*). The sequence is divided into a part that is common to males and females (**a**), a female-specific part (**b**) and a male-specific part (**c**). The DNA binding domain (*OD1*) is *boxed in grey* in the aminoterminal region of **a**. Likewise the oligomerization domain (*OD2*) is *marked in grey* in the carboxyterminal of **a** and extends into the female-specific part (**b**). The sequence encoded by exon m is *underlined* in the male-specific part (**c**)

domain, the male-specific exon 5 is remarkably poorly conserved, displaying only very short stretches of similarity. In *Musca*, the male-specific part of the transcript starts with exon m, introducing an additional 49 aa upstream of exon 5 (underlined in Fig. 2c). This exon appears to be unique to *Musca*, since this sequence

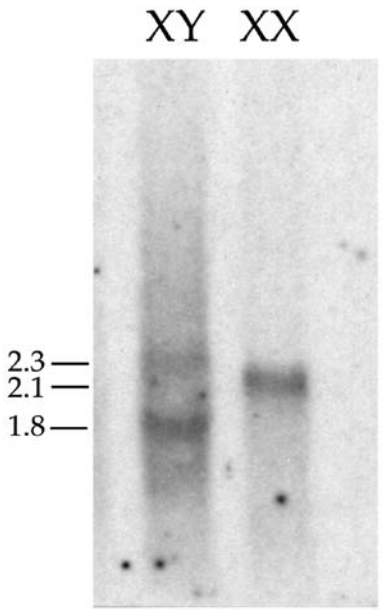

Fig. 3 *dsx* transcripts in adult male (*XY*) and female (*XX*) houseflies. Each lane contains 10 µg poly(A)$^+$ RNA prepared from adult flies and the blot was probed with the fragment *dsx*C (see Materials and methods)

Table 1 Presence of *Md-dsx* splice variants in different genotypes [+ abundant; *(+)* variable, low abundance; – not detected]

	*Md-dsx*M1 (2-3-m-5)	*Md-dsx*F1 (2-3-4)	*Md-dsx*C1 (2-3-m-4)
♀ Adults *(X/X)*	–	+	(+)
♂ Adults *(M^Y/X)*	+	(+)	+
♀ Adults *(M^{III}/M^{III}; F^D/+*	+	+	–
♂ Adults *(M^{III}/M^{III})*	+	–	–
♀ Adults *(F^{man}/F+)*	–	+	(+)
♂ Adults *(F^{man}/F^{man})*	+	(+)	–

is neither present in the male-specific domains of *dsx* in *Megaselia scalaris* (Kuhn et al. 2000) and *Bactrocera tryoni* (Shearman and Frommer 1998) nor in the lepidopteran species *Bombyx mori* (Ohbayashi et al. 2001).

Md-dsx is sex-specifically processed

Northern blot analysis of poly (A$^+$) RNA preparations verified the presence of transcripts of different sizes in male and female RNA samples when probed with an *Md-dsx* sequence common to both sexes (Fig. 3). In adult females, *dsx* produces a prominent transcript of about 2.1 kb, while in males two transcripts can be detected, a prominent band at 1.8 kb and a weak band at 2.3 kb. In addition, we performed a set of RT-PCR reactions with RNA prepared from males and females (listed in Table 1). By and large, we found that products amplified from females were predominantly composed of exons 2-3-4 and are thus likely to correspond to the major 2.1 kb

transcript. Amplification products from males were largely composed of exons 2-3-m-5-6. We therefore assumed this splicing variant to represent the predominant 1.8 kb transcript in males. In addition, our RT-PCR analysis revealed a less abundant splice variant composed of exons 2-3-m-4 which was found in male and female tissues (Table 1). As this variant, due to the incorporation of exon m, is 147 bp larger than the major female splice variant, it may correspond to the weak 2.3 kb transcript in males (Fig. 3). Sequence analysis of the main two splice variants indicates that pre-mRNA of *Musca dsx* is processed in a mode similar to that of *dsx* in *Drosophila*. However, it differs in one important aspect: the donor site of the common exon 3 is joined either to the female-specific exon 4 or to a male-specific exon m located upstream rather than downstream of exon 4 (Fig. 1b). We never detected any amplification products in which exon 3 is directly joined to exon 5 as seen in *Drosophila*.

Given that *Md-dsx* is regulated at the post-transcriptional level in response to absence or presence of *M*, the next question we addressed is whether this regulation is mediated by the switch gene *F*, the proposed principal target of *M* (Dubendorfer and Hediger 1998). To test this, we examined the structure of *Md-dsx* transcripts in strains that either carry the recessive loss-of-function allele of *F*, F^{man}, or the dominant gain-of-function allele of *F*, F^{D}. Houseflies homozygous for F^{man} develop into males even when *M* is absent (Schmidt et al. 1997). These no-*M* males produce only the male type of *Md-dsx* transcripts (Table 1). Evidently, absence of *F* activity results in the male mode of processing. Houseflies of the genotype *M/M;* F^{D}/+ develop into females even in the presence of *M*, because the dominant gain-of-function F^{D} allele overcomes the repression by *M* (McDonald et al. 1978). In these females, we detected *Md-dsx* transcripts of the female 2-3-4 composition (Table 1). Again, it is the state of activity of *F* that determines the splicing mode of *Md-dsx,* irrespective of whether *M* is present or absent. We therefore conclude that *Md-dsx* occupies a position downstream of *F* in the pathway.

The possibility that *Md-dsx* corresponds to *F* seems unlikely for the following two reasons. First, recessive loss-of-function alleles of *F* cause male development, whereas a dominant gain-of-function allele imposes female development. Accordingly, *F* is expected to be active in females but not in males (Dubendorfer et al. 2002; Hilfiker-Kleiner et al. 1993). *Md-dsx*, however, is active in males and females expressing sex-specific functions (see later). Second, genomic sequences around the regulated splice sites of *Md-dsx* in animals carrying either the gain-of-function F^{D} allele or the F^{man} allele did not differ from the corresponding wild-type sequences.

Functional study of *Md-dsx* in *Drosophila*

In *Drosophila*, DSX proteins act as transcriptional regulators that control the activity of genes responsible for the differentiation of sexually dimorphic traits. The sex-specific protein variants DSX^{F} and DSX^{M} behave antagonistically in the regulation of these target genes. For instance, DSX^{M} represses basal transcription of the yolk protein genes, whereas DSX^{F} stimulates transcription by binding to the same upstream promoter sequences (An et al. 1996; Coschigano and Wensink 1993; Erdman et al. 1996). It has been previously demonstrated that ectopic expression of DSX^{F} in XY males counteracts the activity of endogenous DSX^{M} and can impose some attributes of female differentiation (Baker et al. 1989; Waterbury et al. 1999). For instance, XY males carrying a constitutively active *dsxF* transgene contain substantial levels of vitellogenin in the hemolymph and produce a female profile of pheromones (Waterbury et al. 1999). To test whether the gene products of *Md-dsx* are capable of invoking the same responses, we introduced a transgene with *Md-dsx*F1 sequences driven by UAS into *Drosophila* XY males by P-element mediated transposition. When combined with an inducible hsp70-Gal4 driver, these males did not show any sign of sex reversal at the morphological level even when exposed to multiple heat pulses during their development. But, when these males were examined for the presence of vitellogenin, most lines tested gave a clear positive result (Fig. 4a). This demonstrates that *Md-dsx*F1 can overrule repression of yolk protein genes by endogenous DSX^{M} in *Drosophila*. The YP pattern in males though differs not only in levels but also appears less complex than that usually observed in females (Fig. 4a). A simple explanation for this could be differences in the tissue-specific expression of *yp* genes. The strong YP bands observed in female extracts largely derive from egg contents. The YPs in our transgenic males, on the other hand, are exclusively produced by the fat body. It is thus possible that the fat body in these males does not express the full repertoire of YPs.

In a reciprocal experiment, we tested for sex reversing effects by expressing the male variant *Md-dsx*M1 in *Drosophila* XX females. A UAS-*Md-dsx*M1 construct was introduced into XX females carrying the hsp70-Gal4 driver. These animals were exposed to multiple heat shocks during development and were examined as adult flies for the presence of sex transformed structures. Neither the anal nor genital regions were affected nor did this treatment result in the formation of male-specific bristles, the sex comb, on the forelegs. However, some lines displayed variable degrees of male-like pigmentation in the 5th and 6th tergite. The extent of pigmentation was most pronounced in lines carrying *Md-dsx*M1 under the control of an actin5C promoter (Fig. 4b). These lines were derived from a Hermes-based transposition, which is applicable in *Drosophila* (Guimond et al. 2003). Similar results were obtained in previous studies when *Drosophila* DSX^{M} was ectopically expressed using the actin5C promoter (Baker et al. 1989). Pigmentation in the posterior part of the female abdomen is normally repressed by DSX^{F} (Kopp et al. 2000). The presence of $MdDSX^{M1}$ thus appears to be capable of counteracting this repression allowing some level of pigments to be

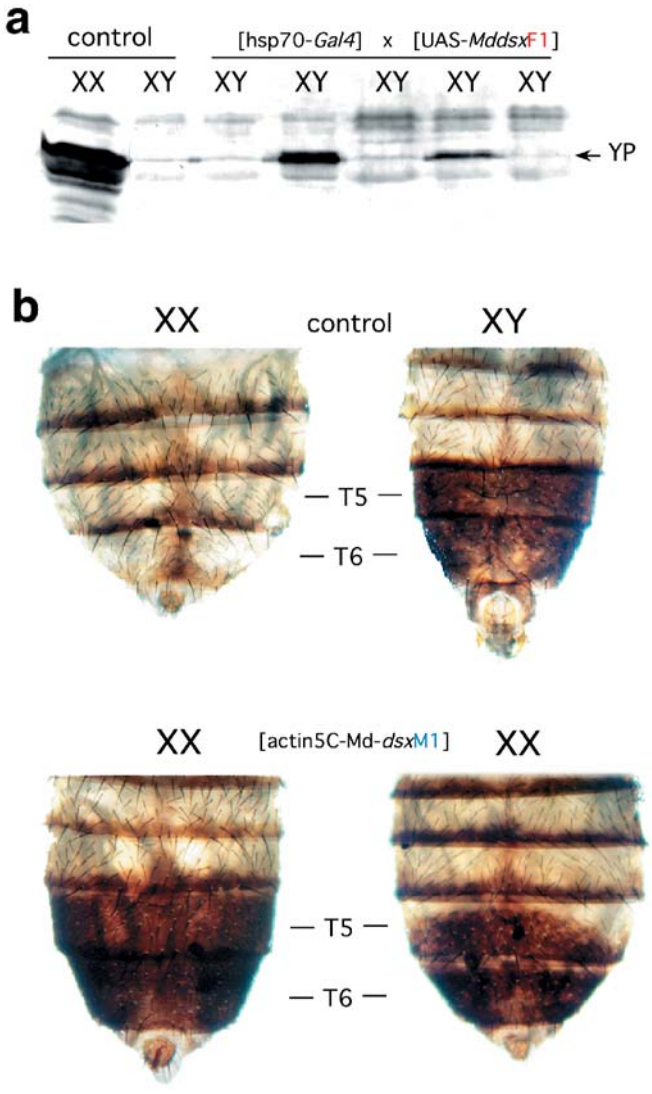

Fig. 4a, b *Md-dsx* expression in *Drosophila*. **a** Effects of *Md-dsx*F under the control of the hsp70 *Drosophila* promoter. Transgenic lines carrying a UAS-*Md-dsx*F1 construct were crossed to hsp70-Gal4 driver lines. Adult flies containing both constructs were exposed to several pulses of heat (1 h at 37°C) before hemolymph was removed for western blot analysis. Hemolymph samples collected from ten individuals were probed with a polyclonal antibody against *Drosophila* vitellogenin. The expected size of yolk polypeptides (*YP*) is indicated by an *arrow*. Controls are non-transgenic flies of the same *white*^{f118} strain. **b** Effects of *Md-dsx*M under the control of the actin5C *Drosophila* promoter. Preparations of abdominal tissues from non-transgenic males and females display the characteristic dimorphic pigmentation patterns in the most posterior tergites, T5 and T6. In transgenic XX individuals male pigmentation can be observed to a variable extent (XX; actin5C-*Md-dsx*M1)

produced. Taken together, these experiments show that forced expression of sex-specific protein variants of *Md-dsx* can elicit sex reverting effects in *Drosophila* suggesting that this gene has a similar function in regulating sexual differentiation in the housefly.

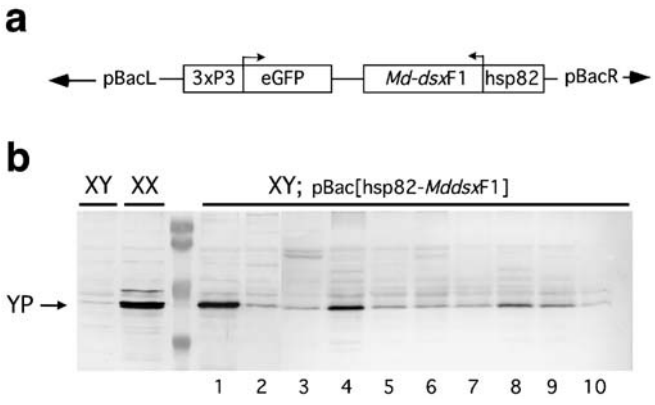

Fig. 5a, b Expression of vitellogenin in *Musca* males carrying the *Md-dsx*F1 transgene. **a** Schematic drawing of the piggyBac construct that was introduced into a *white* strain of *Musca*. The marker EGFP is driven by the 3xP3 promoter described in Horn and Wimmer (2000). *Md-dsx*F1 cDNA has been placed under the control of the promoter of the hsp82 gene from *Drosophila pseudoobscura*. *pBacL* and *pBAcR* indicate the locations of the inverted repeats of the *piggyBac* vector. **b** Western blot analysis of hemolymph from houseflies exposed to several rounds of heat shock (1 h at 42°C). In each lane the hemolymph protein content of an individual fly was electrophoretically separated and probed with a polyclonal antibody against *Musca* vitellogenin

Functional study of *Md-dsx* in the housefly

We generated an expression construct of the female-specific *Md-dsx*F1 cDNA under the control of promoter sequences of the hsp82 gene (Fig. 5a). This cassette was introduced into pBac{3xP3-eGFP}, a vector that was successfully applied to generate transgenic housefly lines in a previous study (Hediger et al. 2001). One line containing a stably integrated copy of this construct was recovered and tested for the presence of female traits in XY males after heat treatment. There was no visible evidence of sexual transformation in the external morphology even after multiple heat shock, but 10% of the transgenic males showed substantial levels of yolk protein in the hemolymph (Fig. 5b). We thus conclude that the *Musca* yolk protein genes are targets of *Md-dsx*. Whether this regulation is direct or indirect remains to be examined. In support of a direct transcriptional control is the presence of sites in the upstream regions of the *Musca* vitellogenin genes which have been shown to bind *Drosophila* DSX protein in vitro (Tortiglione and Bownes 1997).

Loss of *dsx* function in *Drosophila* prevents normal differentiation of dimorphic tissues, and instead leads to the formation of intersexual structures with no clear assignment to either the male or female fate (Baker and Belote 1983). It is expected that disruption of *Md-dsx* causes similar phenotypes in the housefly, if this gene indeed operates in the same pathway. We employed the RNAi technique to disrupt the activity of *Md-dsx*. This method of gene silencing has been successfully applied in *Musca* before (McGregor et al. 2001). dsRNA was prepared from the common region, from the female-

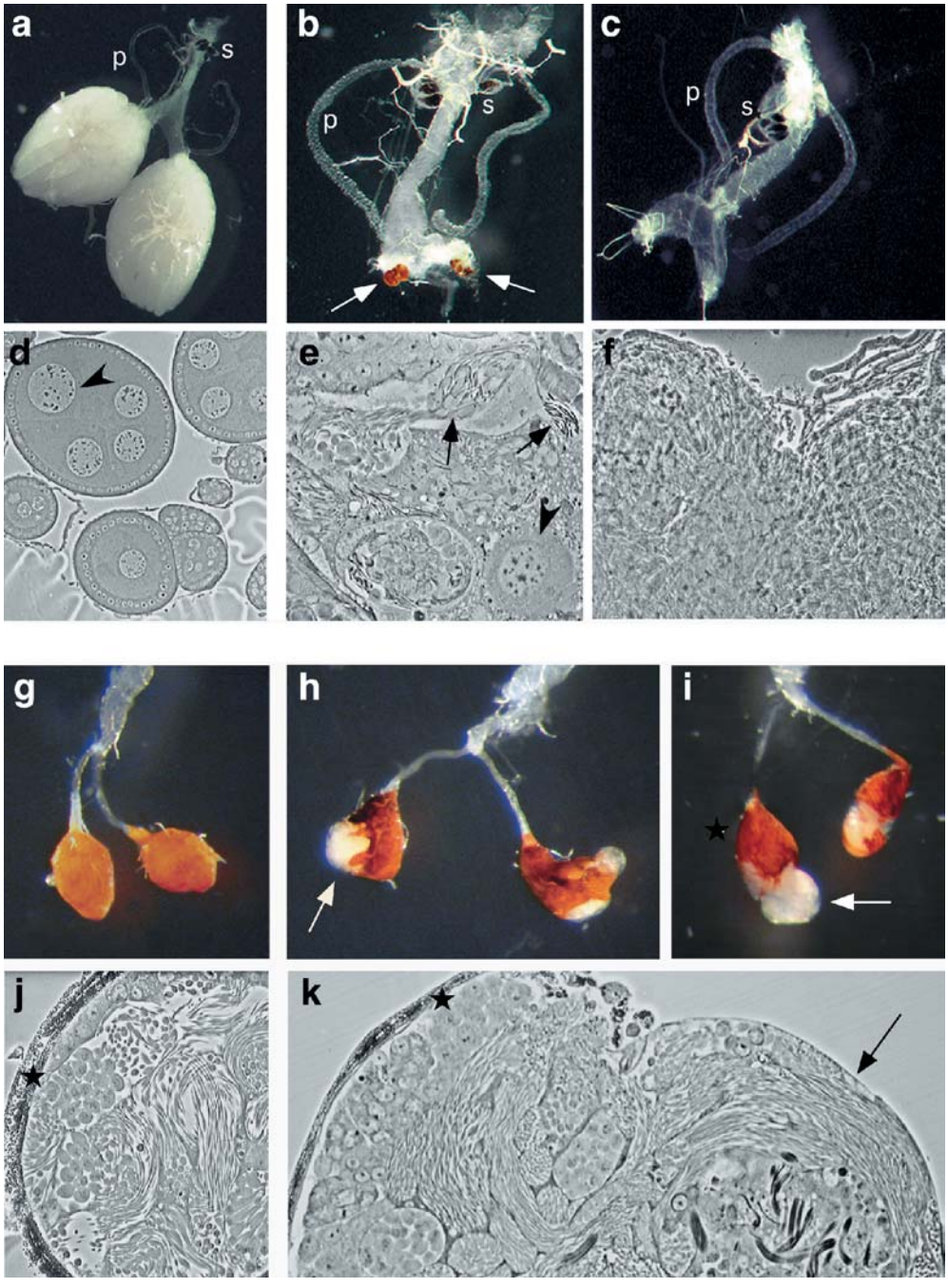

Fig. 6a–k Downregulation of *dsx* by RNAi in early embryogenesis affects gonadal differentiation. **a–f** Ovaries of *M/M; F^D/+* females. **a** A pair of normally developed ovaries of an untreated female (*p* parovarial glands, *s* spermathecae). **b** Markedly underdeveloped ovaries of a dsRNA-treated female. The *arrows* point to gonadal tissue with testis-like pigmentation. **c** In some cases gonadal tissue was completely absent at the tips of the oviducts. **d** Section through normally developed egg chambers. *Arrowhead* points to a polytene nurse cell nucleus. **e** Section through underdeveloped ovaries which are partially surrounded by testis-like tissue. *Arrows* indicate the presence of bundles of differentiating spermatids beneath this tissue. **f** Section through the non-developed ovary shown in **c**. **g–i** Testes of *M/M; +/+* males. **g** Normally developed testes of an untreated male. **h**, **i** Testes removed from males treated with dsRNA of *Md-dsx*. Note the presence of non-pigmented outgrowths at apical and lateral sites of the testis (*arrows*). **j** Section through a normal testis (*star* indicates epithelial tissue). **k** Section through a testis with apical outgrowth (*arrow*)

specific domain of *Md-dsx*F1 and from the male-specific domain of *Md-dsx*M1. Samples of these dsRNA were each injected into either the anterior or the posterior pole of syncytial blastoderm embryos of an autosomal M^{III} strain with marked chromosomes to distinguish genotypically male and female animals (see Materials and methods). About 12% of injected embryos survived to adulthood. A normal 1:1 ratio of males and females was obtained in all of the injected pools. Among these we did not observe any conspicuous abnormalities in the external morphology. Even injections of a concentrated mixture of all three dsRNA samples did not evoke visible phenotypes in adult flies of this strain. We next injected these dsRNA samples into embryos of the *M/M; F^D/+* strain. Again, surviving adults appeared normal in external morphology, but a substantial fraction (27%) of these *M/M; F^D/+* females contained one or two small underdeveloped ovaries with pigmented testis-like tissues located in the apical region (Fig. 6b). In some cases (8%), no gonads were formed at all (Fig. 6c). We examined the cytology of these abnormal ovaries in sections. In many cases, spermatid-like structures were found next to polytene nurse cells, suggesting that the germline content is a mixture of male and female differentiating cells (Fig. 6e).

Table 2 Effects of *dsx* RNAi in *Musca*. The total number of adult flies examined is in parentheses (*yp* yolk protein)

Strain	dsRNA template	♂		♀
		Testes with outgrowth	Yp production	Abberant ovaries
M^{III}/+	dsxC	0% (20)	0% (20)	0% (9)
	dsxF	0% (60)	0% (43)	9% (53)
	dsxM	7% (30)	0% (28)	0% (26)
M^{III}/+; pB{hsp82-dsxF}	dsxC	25% (20)	10% (20)	42% (38)
	dsxF	0% (27)	0% (26)	18% (34)
	dsxM	0% (19)	17% (52)	0% (5)

This intersexual phenotype is specifically caused by *Md-dsx* RNAi, since it was not observed in non-treated females of this strain or in females injected with dsRNA unrelated to *Md-dsx*. Not only the gonads of *M/M;* F^D/+ females appeared to be particularly sensitive to *Md-dsx* RNAi. Also, *M/M;* +/+ males of this strain show abnormal gonadal development after injecting dsRNA. In 47% of the cases we observed non-pigmented outgrowths at the apical ends of testes (Fig. 6h and i). Though sections through these abnormal testes did not reveal the presence of germ cells adopting a female fate (Fig. 6k), spermatid differentiation was clearly compromised, and males were sterile.

To test for sex-specific effects we injected dsRNA prepared from either only female (exon 4) or only male (exons m-5) templates into embryos of the autosomal M^{III} strain. Upon injection of *dsx*F dsRNA, 9% of +/+ females displayed abnormal ovarian differentiation, while testes of M^{III}/+ males were not affected and male fertility was close to normal (Table 2). On the other hand, injections of dsRNA with male-specific *Md-dsx* sequences disrupted testis development in 7% of examined M^{III}/+ males, while ovarian differentiation appeared normal in all cases (Table 2). We noticed that the number of flies with abnormal ovarian differentiation substantially increased when injecting a host strain that, in addition, carried a *Md-dsx*F1 construct driven by hsp82. Now, 42% of the females were affected when injected with dsRNA of the common part of *dsx* and 18% when injected with a dsRNA preparation of the female-specific part (Table 2). Again, injecting dsRNA of male-specific sequences had no visible effect in these females. These results give clear evidence for sex-specific requirements for *Md-dsx* in gonadal development. Together with the data of ectopic expression of *Md-dsx*, they indicate that *Md-dsx* plays an essential role in controlling sexual differentiation of the housefly.

Cis-elements required for TRA/TRA2 mediated activation of the female splice site are present in *Musca dsx*

In *Drosophila*, the female-specific processing of *dsx* depends on the activities of the upstream regulators *tra* and *tra2* (Hedley and Maniatis 1991; Hertel et al. 1996; Ryner and Baker 1991; Tian and Maniatis 1993). The products of these genes bind to *dsx* pre-mRNA to direct the utilization of the splice acceptor site of the female-specific exon 4. This acceptor site is preceded by a polypyrimidine stretch interrupted by several purines and, therefore, considered to be suboptimal for recruiting components of the spliceosome (Hedley and Maniatis 1991; Ryner and Baker 1991). Binding of TRA/TRA2 protein complexes to six 13 nt repeats (dsxRE) in the 3′ untranslated region of exon 4 enables this acceptor site to be recognised and utilized by the generic splicing machinery. In *Md-dsx*, the female-specific splice site is located at the very same position and the 3′ UTR of *Md-dsx* exon 4 contains a cluster of three sequences with substantial similarity to the dsxRE of *Drosophila* (Table 3). Another structural requirement for TRA/TRA2 binding, namely the purine-rich enhancer element (PRE) close to the dsxRE, is also present in the 3′UTR of *Md-dsx* exon 4 (Fig. 7). The dsxREs and the PRE are clustered in a region starting 500 bp downstream of the acceptor site of exon 4 and flanked on both sides by poly(A) signal sequences (arrows in Fig. 7). Amplification of female *Musca* cDNAs with a sense primer in exon 4 and oligo(dT) yielded different fragments that correspond to the sizes expected when poly(A) signals upstream and downstream of the dsxRE/PRE cluster are utilized. This specific arrangement of splice enhancer elements downstream of the regulated splice site suggests that female exon selection depends on an activation mechanism similar to that in *Drosophila*. The polypyrimidine sequence upstream of the activated female splice site, however, neither significantly deviates from the Y_nNYAG consensus nor does it appear suboptimal when compared to the polypyrimidine tract preceding the acceptor site of the male exon 5 (Table 4).

Table 3 Comparison of dsxRE present in the female-specific exon of *Musca dsx*

Drosophila

T C T T C A A T C A A C A
T C T A C A A T C A A C A
T C A T C A A T C A A C A
T C A A C G A T C A A C A

Musca

A C A A C A A T C A A C A
T C A T C A A T C A A C A
T C A A C A A C C A A C A

Table 4 Comparison of splice acceptor sites. Shown are the sequences of the splice acceptor sites upstream of the male-specific exons m and 5 and of the female-specific exon 4 of *Musca domestica* and *Drosophila melanogaster*. Purines in the polypyrimidine tract are *boxed in grey*

```
M. dom. "m" ♂  ----------------ccccctaaacaauuuucuu a cag CCAUACA
M. dom. "5" ♂  ----------------cuuuaucgacuuaccuuca a uag CUACAGA
D. mel. "5" ♂                              uucuguuaucc c cag CUCGAGU

M. dom. "4" ♀  --(582 bp)-------ucucuucuucaauucuguu u uag GACAACA
D. mel. "4" ♀  --(114 bp)-------ucucucgaucugaucuaaa c cag GCCAAUA

CONSENSUS                          YYYYYYYYYYYYYYYYY N CAG
```

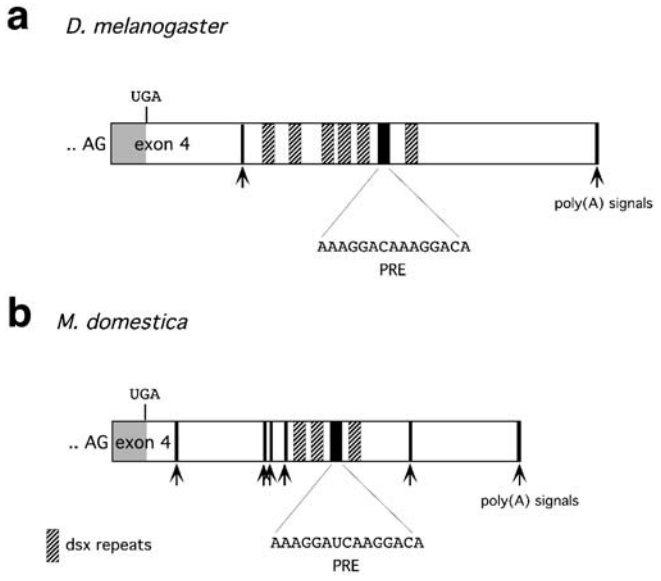

Fig. 7 Distribution of *dsx* repeats in the female-specific exons of *Drosophila* (**a**) and *Musca* (**b**). Translational stops (*UGA*) and potential poly(A) sites are indicated. Location of the 13 nt *dsx* repeats are marked as *hatched boxes*, and the purine-rich sequence (*PRE*) as a *filled box* in the 3′UTR

Another significant deviation from the *Drosophila* situation is the existence and differential splicing of an additional exon m upstream of the female-specific exon 4. This sequence is only included in the two male splice variants, which argues against a simple mechanism in which female-specific splicing of *Md-dsx* is based only on activation of the female acceptor site. The polypyrimidine tract preceding exon m is interrupted by several purines and deviates more significantly from the Y_nNYAG consensus than the polypyrimidine tract of exon 4 (Table 4). No potential TRA/TRA2 binding sites have been found in the vicinity of this exon.

Discussion

We have isolated and characterized a gene in the housefly *M. domestica* with structural and functional homology to the sex-determining gene *dsx* in *D. melanogaster*. The functional correspondence was demonstrated by misexpression studies in *Drosophila* and *Musca*. The female-specific variant, $MdDSX^F$, induces yolk protein synthesis, a typical female physiological response, when expressed in *Drosophila* and *Musca* males. The male variant $MdDSX^M$ causes male-like pigmentation of the posterior tergites in *Drosophila* females. Apart from these subtle sex-reversing effects, the transgenic flies remained morphologically normal. The failure of these transgenes to induce complete sex reversal can be attributed to the antagonistic activity of endogenous *dsx*. A previous report (Waterbury et al. 1999) demonstrated that the final sexual phenotype depends on the relative amount of DSX^M and DSX^F expressed in *Drosophila* cells. For instance, XY individuals expressing DSX^F from a transgene can be gradually transformed into pseudofemales when the dose of endogenous *dsx*, which expresses DSX^M, is decreased. Hence, the female-promoting activity of $MdDSX^F$ in transgenic *Musca* males may be antagonized by the two copies of intact endogenous *Md-dsx* that produce $MdDSX^M$. This explains why these transgenic males are fertile and do not display any detectable female traits apart from the presence of yolk in the hemolymph. It appears that, like in *Drosophila*, genes expressing physiological traits are more responsive to changes in the relative amounts of DSX^M and DSX^F than genes which express morphological traits (Baker et al. 1989; Waterbury et al. 1999). The same observation was made in the lepidopteran species *Bombyx mori* (Suzuki et al. 2003). The female-specific activity of the *dsx* homologue, *Bmdsx*, elicits a physiological female response, namely synthesis of vitellogenins and hexameric storage proteins, and downregulation of pheromone-binding proteins that are preferentially expressed in males, but does not result in a morphologically visible female transformation in *Bombyx* males (Suzuki et al. 2003).

Regulation of yolk protein genes in *Musca*

In standard *Musca* strains, the three yolk protein genes (*Mdyp1*, *Mdyp2*, *Mdyp3*) are transcriptionally repressed in males (White and Bownes 1997). It is thus conceivable that ectopic $MdDSX^F$ in males relieves this repression by a direct interaction with the promoter of these genes. In *Drosophila*, transcriptional regulation of yolk protein genes is the best characterized function of *dsx*. Both variants, DSX^F and DSX^M, can bind to sequences in the promoter region of the yolk protein genes, *yp1* and *yp2*

(Burtis et al. 1991; Coschigano and Wensink 1993). A zinc finger-like DNA-binding domain in the common part of the DSX polypeptides is responsible for binding to several sites in the fat body enhancer (FBE; Erdman and Burtis 1993). Though both proteins bind to the same enhancer, they elicit opposite responses: DSX^F binding results in activation, and DSX^M binding in repression of transcription (An and Wensink 1995a, 1995b). This difference in molecular behaviour is defined by the second oligomerization domain (OD2) which interacts with distinct sets of cofactors (An et al. 1996). Our results suggest that *Musca* DSX proteins have a conserved function in *yp* gene regulation. First, both *Musca* variants share a DNA binding domain (OD1) that is almost identical in amino acid sequence to that of *Drosophila*. Second, in *Drosophila*, $MdDSX^F$ can relieve repression of *yp* transcription imposed by endogenous DSX^M. This is most likely achieved by effective competition for the same binding sites in the FBE. To activate *yp* transcription, $MdDSX^F$ must be capable of interacting with cofactors in *Drosophila* that normally bind to DSX^F. The high degree of sequence conservation found in OD2 of $MdDSX^F$ and DSX^F suggests that this oligomerization domain is responsible for these specific interactions.

A direct role of *Md-dsx* in regulating transcription of the *Musca yp* genes is supported by the presence of potential *dsx* binding sites in the promoter regions of *Mdyp1* and *Mdyp3* (Tortiglione and Bownes 1997; C. Siegenthaler, unpublished results). Furthermore, some of the sites in *Mdyp1* were able to bind *Drosophila* DSX in gel-mobility shift assays (Tortiglione and Bownes 1997). Given the high structural similarity of the DNA binding domain, it seems likely that these sites can also interact with MdDSX. None-the-less, the finding that the promoter sequences of *Mdyp1* cannot confer sex-specific expression in *Drosophila* questioned the contribution of *Md-dsx* in controlling yolk protein synthesis (Tortiglione and Bownes 1997). The authors proposed that, different from *Drosophila* where *dsx* is the primary determinant, *Musca* involves the endocrine system and uses ecdysteroids as a key regulator for sex-specific expression of YP. This mode of hormonal control allows the cyclical laying of eggs to be synchronized with cyclical synthesis of YP. Accordingly, females exhibit a distinct peak of ecdysteroid concentration during egg cycles, whereas the level in males remains continuously low (Agui et al. 1985). The strong correlation between levels of ecdysteroids and YP suggested that *Md-dsx* plays no or only a marginal role in the control of YP expression. However, we demonstrated that expression of $MdDSX^F$ is sufficient to elicit YP synthesis even in *Musca* males where ecdysteroid levels remained low (C. Siegenthaler, unpublished results). We therefore propose that *Md-dsx* is an integral component and interacts with ecdysteroids in this regulation. Binding of MdDSX may influence the response threshold of *yp* genes to ecdysteroids, e.g. binding of $MdDSX^F$ may lower the threshold to a level where even the low ecdysteroid concentration in males is sufficient to trigger expression of the *yp* genes.

Role of *Md-dsx* in gonadal differentiation

Homologues of *dsx* are found in a growing number of insect species (Kuhn et al. 2000; Ohbayashi et al. 2001; Pane et al. 2002; Shearman and Frommer 1998). In all reported cases, it has been shown that the *dsx* homologue produces sex-specific mRNA variants suggesting that *dsx* is widely used as a double switch in the sex determination pathway. Our RNAi study provides evidence that *Md-dsx* is indeed involved in important aspects of dimorphic development. Interference of *Md-dsx* in embryos causes abnormal differentiation of gonads in males and females. This result did not come as a surprise as correct assignment of gonadal soma to become either testes or ovaries is likely to be one of the first implementations of the sex-determining program. Hence, loss of *dsx* at an early stage is expected to disrupt normal gonadal differentiation and to cause ambiguity with regard to the sexual fate of this tissue. The occurrence of pigmented testicular-like tissue in gonads of RNAi-treated females can thus be explained as a direct result of this ambiguous state. Likewise, the unpigmented outgrowths found in the gonads of RNAi-treated males may have derived from cells with ambiguous or unspecified assignment. Intersexual and undifferentiated tissues are typical hallmarks of the phenotypes described in *dsx* mutant *Drosophila* flies (Nothiger et al. 1987). The mutation also affects the internal reproductive system in *Drosophila* females (Hildreth 1964). Often small degenerated ovaries were found in XX; *dsx*/*drx* animals similar to those that we observed in *dsx* RNAi-treated *Musca* females. In line with a sex-specific activity of *Md-dsx*, the specific repression of male or female messages led to abnormal gonadal differentiation only in the affected sex. We infer from this that *Md-dsx* is essential for the correct sexual development of embryonic gonads.

Injections of *Md-dsx* dsRNA in embryos left the external morphology of the developing adults unaffected. This does not necessarily mean that *Md-dsx* does not control the sexual differentiation of the imaginal cells. Rather, the injected material may not persist into later developmental stages, and therefore may not interfere with intrinsic *dsx* RNA. Genetic studies in *Drosophila* have unambiguously demonstrated that *dsx* does also control the sexual differentiation of imaginal disc cells in advanced developmental stages (Baker and Ridge 1980; Belote and Baker 1982).

Regulation of *Md-dsx*

The production of sex-specific transcripts in *Drosophila* is achieved by differential splicing. In males, exon 4 is skipped by default, and instead the downstream exons 5 and 6 are included in the mature transcript. In females, the presence of the splice regulatory activities of TRA/TRA2 promotes the incorporation of exon 4 in the mature transcript. This pattern of sex-specific splicing is also observed in the Queensland fruitfly *Bactrocera tryoni* and

the Phorid fly *Megaselia scalaris* where it occurs in equivalent positions of the corresponding *dsx* genes (Kuhn et al. 2000; Shearman and Frommer 1998). Moreover, Pane et al. (2002) demonstrated in a recent report that female-specific splicing of the *dsx* gene in the Mediterranean fruit fly *Ceratitis capitata* depends on the activity of the *tra* homologue. The presence of putative TRA/TRA2 binding sites in the female-specific exon of *dsx* in *Bactrocera* and *Megaselia* gives further support to the notion that female exon selection by activation is common in dipteran insects. In the lepidopteran species *Bombyx mori*, the *dsx* homologue is subjected to the same pattern of sex-specific processing, but the underlying mechanism appears to be different (Suzuki et al. 2001). Here, female splicing represents the default mode when tested in HeLa nuclear extracts, and also the female exon is devoid of putative TRA/TRA2 binding sites. The authors therefore proposed that the female exon is selectively repressed in male silk moths by a yet unknown mechanism (Suzuki et al. 2001).

The situation in *Musca* is more consistent with the activation mode, primarily because putative splice enhancing sequences are present in the female exon. However, the existence of another differentially spliced exon upstream of the female exon adds a level of complexity not observed previously. Its absence in female transcripts suggests that an additional level of control exists which selectively prevents the recognition of exon m in female cells. It is feasible that activation of the female splice acceptor and repression of exon m are mediated by the same mechanism. For instance, sex-specific processing of the *tra* homologue in *Ceratitis* involves 5′ and 3′ splice site selection and exon skipping (Pane et al. 2002). The clustering of several TRA/TRA2 binding sites in the vicinity of these regulated splice sites suggests that *Ceratitis* TRA has an autocatalytic function which is capable not only of activating splice sites but also of repressing splice sites in the *tra* pre-mRNA. Furthermore, the autoregulatory activity of TRA2 in spermatogenesis of *Drosophila* mediates repression of a specific splice site rather than its activation (Chandler et al. 2003; Mattox and Baker 1991). On the other hand, it is also possible that differential processing of exon m is uncoupled from that of the female exon. Instead, it may be omitted by default but become specifically activated in male cells. In particular, the poor match of its 3′ splice site to the polypyrimidine consensus lends some support to this idea. In this scenario, correct processing of *Md-dsx* may thus not only rely on the use of female-specific splice activators but also on male-specific activators.

Evolution of sex determination pathways

The existence of different sex-determining mechanisms in natural populations of *M. domestica* makes the housefly a particularly suited system for studying evolutionary changes in sex determination pathways (Dubendorfer et al. 1992). We believe that these variations reflect minor changes in an otherwise well conserved pathway. By identifying the genes in the *Musca* pathway, we aim at an understanding of the principles of the underlying genetic control and, by comparison with sex-determining genes in other species, of how such pathways evolve. Thus far, our results are consistent with the model proposed by Wilkins (1995) that sex determination pathways evolve from bottom to top in a retrograde fashion. *dsx* appears to be a common terminal regulator in all hitherto analysed pathways. But this extent of congruence seems to halt at the level of the upstream regulator *F*. While, in *Musca*, this gene seems to be the direct target of the primary signal, *Drosophila* recruited yet another upstream switch, *Sxl*. The reason for this added level in the cascade is not known. A sex-determining function of *Sxl* has so far only been demonstrated in members of the genus *Drosophila*, and its recruitment to the pathway is thus believed to be a rather recent event (Schutt and Nothiger 2000). This level of understanding may prove helpful for the identification of corresponding sex switches in other insects, in particular in species of medical and agricultural relevance. This work will contribute not only to an understanding of the evolutionary forces that shape sex-determining pathways, but also to the design and application of new genetic tools for a use in population control programs of pest insects.

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4.2 Yolk protein synthesis in Musca domestica is controlled by ecdysteroids and *dsx* proteins.

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in preparation

Yolk protein synthesis in *Musca domestica* is controlled by ecdysteroids and *dsx* proteins

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Abstract

Synthesis of yolk proteins (YP) in *Musca domestica* appears to be regulated by sex-specific differences in the concentration of ecdysteroids. In females, the ecdysteroid level follows a cycle that correlates with the cyclic expression of YP, whereas in males, the ecdysteroid titre is constantly low. Injection of 20-hydroxy-ecdysone (20E) into males induces YP synthesis. In this work, we show that non-hormonal factors are also involved in controlling the expression of YP. Musca males of certain strains express YP, even though their ecdysteroid titre is not elevated. These males express low levels of the female splice variants of *Musca domestica doublesex.* The *doublesex* gene is known to control directly the transcription of the *yp* genes in the fat body of *Drosophila melanogaster*. Here, we provide evidence that the Musca homologue, *Md-dsx,* contributes to the sex-specific regulation of YP expression.

Keywords

Musca domestica, yolk proteins, *doublesex*, ecdysteroids

Introduction

Yolk proteins (YP) are the nutritional basis for the developing embryo in all egg-laying species. In insects, YP are synthesized in the female fat body, secreted into the hemolymph and then accumulate in the growing oocytes. In some insect species, such as Drosophila and Musca, YP are also produced by the follicle cells surrounding the growing oocyte (Brennan *et al.*, 1981; Jowett and Postlethwait, 1980; DeBianchi *et al.*, 1985). Three YP genes have been identified in *Musca domestica*: *Mdyp1*, *Mdyp2* and *Mdyp3*. MDYP1 and MDYP3 share the highest degree of amino acid similarity (82.5%), whereas MDYP2 reveals only 58.9% similarity to MDYP1 and 63.5% to MDYP3. Transcripts of all three *Mdyp* genes can be found in the fat body and in the ovary of Musca females (White and Bownes, 1997). YP production occurs in cycles in species where oogenesis and egg deposition are also cyclic. In contrast, if

oocyte maturation and egg laying are continuous, as in Drosophila, YP are synthesized at a constant rate (Bownes, 1989). In Musca, oocytes develop synchronously, and after about five days, they are laid in a batch. Adams (1974) described ten oocyte stages: Stages 1 to 3 are the previtellogenic stages when the oocytes do not yet contain any yolk. Stages 4 to 8 are the vitellogenic stages, characterized by the oocyte taking up the YP synthesized by the follicle cells and the fat body. Stages 9 and 10, finally, are the postvitellogenic stages, when YP uptake has stopped, the nurse cells are degenerating and the egg is ready to be laid.

Yolk protein synthesis can be controlled cell-autonomously by genes of the sex determination cascade. In Drosophila, the terminal regulator of the sex determination cascade, the *doublesex (dsx)* gene, directly controls transcription of the *yp* genes in the fat body (Coschigano and Wensink, 1993). In other species, YP were shown to be controlled by a sex-specific hormonal regimen, in particular by juvenile hormone and ecdysteroids (Izumi *et al*., 1994). A burst of ecdysteroids after a blood meal, for instance, triggers YP production in females of *Aëdes aegypti* (Dhadialla and Raikhel, 1994).

Hormones play also an important role in controlling YP expression in *Musca domestica*. One hormone that seems to be involved is the juvenile hormone (JH), which is produced in the corpora allata-corpora cardiaca complex. Removal of this complex causes YP expression in females to drop to a low level; when these females are then supplied with JH or methoprene, a JH analogue, YP production is resumed (Agui *et al.*, 1985; Adams and Filipi, 1988). While application of methoprene cannot stimulate YP synthesis in males (Agui *et al.*, 1985; Agui *et al.*, 1991; Adams *et al.*, 1989), injection of 20-hydroxy-ecdysone (20E), the active isoform of ecdysone, induces YP synthesis in both males and females, even when JH production is eliminated by removing the corpora-allata complex (Agui *et al.*, 1991). Thus, it appears that JH is rather a permissible factor in the regulation of YP synthesis, but not a controlling agent. In *Aëdes aegypti*, fat body cells require to be exposed to high JH levels before ecdysteroids can trigger vitellogenin synthesis (Zhu *et al*., 2003). In Musca, JH may serve a similar function to make fat body cells competent to respond to high ecdysteroid levels with the production of YP.

The main control agents of YP production in Musca are the ecdysteroid hormones. In females, the concentrations of ecdysteroids and YP in the hemolymph correlate, both reaching a maximum at oocyte stages 6 to 7, rising and dropping simultaneously (Fig. 1; Adams and Filipi, 1983; Adams *et al.*, 1985; Agui *et al.* 1985). In addition, transcription of the *Mdyp* genes follows the same cyclic pattern: mRNAs of *Mdyp1* and *Mdyp3* are maximally abundant in the fat body and in the ovary at oocyte stages 4 to 8, while *Mdyp2* transcripts show a maximum at stages 5 to 9 (White and Bownes, 1997). Peak ecdysteroid levels in females have been found to be variable, depending on the Musca strains used; concentrations between 18pg/µl (Agui *et al.*, 1985) and 50pg/µl (Adams *et al.*, 1985) were measured. The situation in males is different, as the ecdysteroid level remains continuously low at about 5pg/µl (Agui *et al.*, 1985). Injection of 20E into males induces transient YP expression (Adams *et al.*, 1989). Yet, ecdysteroids are apparently not the only factor required for regulating YP synthesis in Musca. In ovaryectomized females, ecdysteroids in the hemolymph drop to a very low, male-like level (< 4pg/µl), but these females nevertheless continue to produce YP, if only at a low rate (Agui *et al.*, 1985). Furthermore, both allatectomized females and males start to produce YP after injection of 20E, but males are about 100 times less sensitive, and the response is delayed by a two-fold (Agui *et al.*, 1991).

Thus, it seems that in females other factors must be present, rendering the *Mdyp* genes receptive towards activation by ecdysteroids. In this report, we demonstrate that *Md-dsx* is a likely candidate. In Drosophila, *dsx* is known to directly control the expression of the three *yp* genes in the fat body: The male splice form, DSX^M, represses basal transcription, while the female form, DSX^F, enhances it (Coschigano and Wensink, 1993). The Musca homologue of Drosophila *dsx* has recently been isolated, and two sex-specific splice forms were found, *Md-dsx*^{F} and *Md-dsx*^{M} (Hediger *et al.*, 2004; Fig. 5). As in Drosophila, the *Md-dsx* gene is the terminal regulator of the sex-determining cascade, relaying the primary sex-determining signal to the sex-differentiating genes, such as the yolk protein genes, which implement the selected fate.

The primary signal in Musca sex determination is the male-determining factor *M* (for a review, see Dübendorfer *et al*., 2002*)*; when present, it represses the female determining key gene *F*, which in turn leads to *dsx* being spliced in the male mode, dsx^M. Absence of *M,* along with the presence of maternal *F* product, allows the activation of the zygotic *F* gene (Dübendorfer and Hediger, 1998), and consequently, *dsx* is differentially spliced to give rise to the female form of the protein, DSX^F (Fig. 2; Hediger *et al*., 2004). *M* factors can be found on the Y chromosome, or on any of the five autosomes and even on the X chromosome in different strains (Rubini *et al*., 1972); not all the *M* factors are fully equivalent as some of them are weaker than others. For instance, M^I (*M* on chromosome I) allows the production of YP in otherwise normal and fertile males (Schmidt *et al.*, 1997). The gene *F* is located on chromosome IV, and two mutant alleles are known. One is a loss-of-function allele, F^{man} ($F^{masculinizer}$); individuals homozygous for F^{man} develop as males, independently of an *M* factor, whereas females in this strain have the genotype F^+/ F^{man}. Since F^{man} males synthesize YP, Schmidt *et al.* (1996) suspected some residual *F* activity and concluded that F^{man} is not a null allele, but rather a strong hypomorph (Fig. 2, right panel).

In this report, we demonstrate that ectopic expression of YP in M^I and F^{man} males correlates with misregulation of *Md-dsx*, suggesting that this gene contributes to the control of *yp* genes in Musca.

Materials and methods

Fly strains

A wildtype, a white (w/w) and a multiply marked strain (*ac*/*ac*; *ar*/*ar*; *bwb*/*bwb*; *ocra*/*ocra*) were used as standard strains. These strains have standard-type sex determination: XY males and XX females. The autosomal markers were: Chromosome I, *ac* – ali curve, curved wings; chromosome II, *ar* – *aristapedia*; chromosome III, *bwb* – *brown body*, *w* – *white*, white eyes; chromosome V, *ocra* – *ocra*, ochre eyes.

M^I strain: Male genotype XX; M^I +/+ *ac*; *ar*/*ar*; *bwb*/*bwb*; *ocra*/*ocra*. Female genotype XX; *ac*/*ac*; *ar*/*ar*; *bwb*/*bwb*; *ocra*/*ocra*

F^{man} strain: Male genotype: XX; *ac*/*ac*; F^{man}/F^{man}. Female genotype: XX; *ac*/*ac*; F^{man} Ba^{+}/F^{+} *Ba* (*Ba* – *bald abdomen*, chromosome IV).

Strains were reared as described previously (Schmidt *et al.*, 1997).

SDS-PAGE and western blotting

Hemolymph samples of individual flies were collected by inserting a glass capillary into the ventral thorax. The samples were transferred into 13µl of 2x SDS sample buffer on ice, boiled for 5min and stored at –78°C. Oocyte stages in females were determined after taking the hemolymph samples, using the definitions of Adams (1974, see introduction). Ovaries and fat body: Flies were dissected in Musca Ringer's solution (7.5g/l NaCl, 1g/l KCl, 0.18g/l $CaCl_2 \cdot 2H_2O$, 0.12g/l $NaHCO_3$, pH 7); ovaries and/or fat body were homogenized in 20µl 2x SDS, and insoluble material was removed by centrifugation. The supernatant was boiled for 5min, and the samples were stored at –78°C. SDS-PAGE was carried out using the BioRad MiniProtean II System; entire samples (hemolymph) or 5µl (ovary and fat body samples) were loaded on a 12% SDS gel and separated by electrophoresis. Proteins were transferred to a 0.45µm nitrocellulose membrane (BioRad) in Tris-Glycin-Methanol. The membranes were incubated in blocking solution (4% low fat milk powder in TBS/0.05% Tween). The primary antibody (polyclonal anti-yolkprotein antibody, kindly provided by Dr. T. Adams) was diluted 1:20'000 in TBS/0.05% Tween with 1mg/ml BSA; membranes were incubated in the antibody solution for 1h at room temperature. The antigen-antibody complex was detected using the alkaline phosphatase (AP) conjugated anti-rabbit antibody by Promega at a dilution of 1:7'500 in TBST/1mg/ml BSA.

RIA

Hemolymph samples were taken as described above and pooled on ice. The volume of the samples was measured using a micropipette. The pooled samples were dried in a SpeedVac for 45min. The hemolymph samples were exhaustively extracted with 60 p.c. methanol. Aliquots of the extract were subjected to RIA. High avidity (20,000 fold) rabbit antiserum, raised against 20-hydroxyecdysone-6-ketoxime thyroglobulin conjugate, was used. RIA measurements were performed using a protocol described earlier (Maroy *et al.*, 1988), but with overnight incubations at 4°C. Results are expressed in 20-hydroxyecdysone equivalents, and normalised to hemolymph volume (µl).

Northern blot analysis

Total RNA of 250mg flies (~18 adult males or ~15 adult females) was extracted with the AGPC technique (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated using the Oligotex mRNA Maxi Kit (Qiagen). 1µg of mRNA per lane was fractioned by formaldehyde agarose gel electrophoresis, transferred to Hybond-N+ nylon membrane (Amersham) by blotting with 10x SSPE and cross-linked in a UV Stratalinker 2400 (Stratagene). Hybridizations were carried out in formamide hybridization solution at 42°C using $6 \cdot 10^6$ cpm of labelled probe. Labelled antisense RNA probes were generated by in vitro transcription of PCR fragments of the *Mdyp* genes using T7 RNA polymerase (Promega) and α-^{32}P-rCTP (Amersham). After exposure, the filter was kept at –78°C for several months and then reprobed with RNA probes of the other *Mdyp* genes.

Injection of 20E

20-hydroxy-ecdysone (Sigma) was diluted in Musca Ringer's solution to concentrations of 10ng/µl and 1µg/µl. 1µl of these solutions was injected with a glass needle into the abdomen of 3d old males of the M^I strain and of a standard strain as a control. Hemolymph of the injected flies was taken 24h after injection and analyzed by western blotting as described above.

RT-PCR

Poly(A)$^+$ RNA was prepared as described above. 0.5µg mRNA was retro-transcribed using Enhanced AMV Reverse Transcriptase (Sigma), following the manufacturers protocols. Male and female transcripts of *dsx* were amplified from cDNA by standard PCR techniques using Taq DNA polymerase (Promega). Primers used for dsx^F: Primer C in the common exon 3, primer F in the female exon 4. Primers used for dsx^M: Primer C (common exon 3) and primer M in the male exon 5 (Fig. 5). Samples of 5µl were taken after 24, 27, 30 and 39 PCR cycles and analyzed on a 1% agarose gel.

Results

Mdyp1 and *Mdyp3* are expressed in males of M^I and F^{man} strains

It has previously been shown that some males of certain *Musca* strains produce yolk proteins (Schmidt *et al.*, 1997). We are currently keeping two "YP-strains" in our lab, namely the M^I strain and the F^{man} strain. In the M^I strain, males carry the male-determining factor *M* on chromosome I; about 80% of M^I males produce yolk proteins, but apart from that, they are

morphologically perfect and fertile males. F^{man} males are homozygous for F^{man}, a hypomorphic allele of the female-determining gene *F*. In F^{man}/F^{man} males, YP production seems to vary between the generations: from only 4% of the males to 40%; additionally, the fertility in F^{man} males is slightly reduced due to behavioural defects (S. Käppeli, personal communication). We suspect that these two phenomena are caused by some residual *F* activity of the F^{man} allele.

We analyzed YP synthesis in males of these two strains and in males and females of a control strain (XY males, XX females). Yolk proteins in hemolymph, ovaries and fat body of 3-day-old males and 2-5 day old females were detected by western blotting and subsequent immunostaining. As expected, standard males, regardless of their age, never contained any trace of YP in their hemolymph and fat bodies (Fig. 3a). In standard females, YP concentration depends on the stage of ovarian development. Only weak signals were seen in females with pre- and postvitellogenic oocytes (stages 1-3 and 9-10), whereas during the vitellogenic stages (4-8), YP levels in the hemolymph reached their maximum. Three bands of YP were detected in hemolymph, fat body and ovaries. These results correspond to those of Agui *et al.* (1985) and Adams and Filipi (1983). Additionally, we found that the slowest migrating YP was exclusively present in vitellogenic females (oocyte stages 4-8), and that its concentration was generally lower than that of the duplet proteins. Agui *et al.* (1991) and White and Bownes (1997) observed a similar cycling in the concentration of *Mdyp* transcripts, and White and Bownes (1997) showed, in addition, that *Mdyp2*-mRNA is only detected in vitellogenic females. We thus concluded that the slowest migrating YP in females corresponds to MDYP2.

Females of the M^{I} strain (Fig. 3b) exhibit the same quantity and pattern of YP in hemolymph, in ovaries and in the fat body as control females of a standard XX strain (Fig. 3a). Levels of YP expression in males of the M^{I} and F^{man} strain vary considerably. First, the amount of YP in the hemolymph can range from undetectable to nearly female levels in different individuals of the same strain and of the same age (Fig. 3b). Second, we found no evidence for cyclic YP expression; instead, YP concentration in the hemolymph gradually

increased with age. No YP are detectable in the M^{I} male fat body. This can be explained by a low synthesis rate of YP in the fat body and subsequent accumulation in the hemolymph, since males have no ovaries and therefore lack a "sink" for the secreted YP. The third and most striking difference is the absence of the slowest migrating YP, MDYP2, in male hemolymph even when the concentration of the other two proteins, MDYP1 and MDYP3, is as high as in vitellogenic females.

To test whether *Mdyp2* is transcribed in YP-synthesizing males, we performed northern blot analysis of *Mdyp* transcripts and observed that *Mdyp2* transcripts were absent in M^{I} males, while transcripts of *Mdyp1* and *Mdyp3* are clearly detectable (Fig. 3c). As expected, standard XY males do not transcribe any of the *Mdyp* genes, whereas XX females show strong signals of all three *Mdyp* transcripts. Additionally, in M^{I} and in standard males, a slower migrating transcript was observed that hybridized with the *Mdyp3* probe. Thus, *Mdyp2* is expressed only in females and only during the vitellogenic oocyte stages 4-8, while YP synthesized from *Mdyp1* and *Mdyp3* are also found in pre- and postvitellogenic females (White and Bownes, 1997) and in M^{I} and F^{man} males (this work). *Mdyp2* transcripts and MDYP2 proteins were found not only in the ovaries, but also in the fat bodies of females. Hence, lack of *Mdyp2* expression in M^{I} and F^{man} males cannot be explained by the absence of ovaries.

Expression of *Mdyp2* depends on a high level of ecdysteroids

Females produce MDYP2 only when the ecdysteroid level reaches its maximum suggesting that *Mdyp2* is only be expressed when the ecdysteroid concentration is high. To test this hypothesis, we induced YP synthesis in males by injecting 20-hydroxy-ecdysone and analyzed the YP expression pattern in the hemolymph by western blotting. We found that 10ng of injected 20E was sufficient to induce expression of *Mdyp1* and *Mdyp3*, whereas a 100fold higher dose, 1µg, was necessary to induce *Mdyp2* (Fig. 4). *Mdyp2* was induced in standard XY and in M^{I} males, which showed that the *Mdyp2* gene of M^{I} males is intact.

Ecdysteroid levels are not increased in YP synthesizing males

YP synthesis in M^{I} and F^{man} males may be due to elevated ecdysteroid levels, high enough to induce MDYP1 and MDYP3, but not enough for the induction of MDYP2.

To test this possibility, we analyzed pooled hemolymph samples of 100-250 3d old flies of each genotype. A radio-immuno assay (RIA) revealed that the ecdysteroid concentration was not increased in M^{I} and F^{man} males compared to XY standard males (Table). However, the ecdysteroid level in females of the non-standard strains was slightly reduced relative to XX standard females. This difference is very likely strain-specific, and, since earlier publications have already shown variation in ecdysteroid titres between strains (Agui *et al.*, 1985: 18pg/µl; Adams *et al.*, 1988: 50pg/µl), probably irrelevant.

Males that produce YP express *dsx*^{F}

Since ecdysteroid levels are not increased in M^{I} and F^{man} males, other factors must be responsible for YP expression. The Musca *dsx* gene is a likely candidate. The YP genes are direct targets of *Dm-dsx* in the fat body of Drosophila, and *Md-dsx* is a homologue of *Dm-dsx*. In Musca strains with standard sex determination, *Md-dsx* pre-mRNA is spliced into two sex-specific variants; dsx^{M} is present in males, whereas dsx^{F} is found exclusively in females (Hediger *et al.*, 2004). It is possible that *Md-dsx* is misregulated in the F^{man} and the M^{I} strain.

We performed a semi-quantitative RT-PCR for levels of *Md-dsx*^{F} and *Md-dsx*^{M} transcripts on mRNA from males and females of the M^{I} and F^{man} strain and of a standard XX/XY strain. Levels of *Md-Sxl* were used as an internal standard. Primers used for detection of the sex-specific splice variants of *Md-dsx* are shown in Fig. 5, and the results are summarized in Fig. 6. *Md-Sxl* signals were of about the same intensity in all genotypes examined, indicating that there was no significant difference in quality and concentration of the different mRNA samples. The experiment confirmed that standard XY males express abundant levels of *Md-dsx*^{M}, but no *Md-dsx*^{F}, whereas females of all strains express *Md-dsx*^{F} at high levels. Transcripts of *Md-dsx*^{M} were also found in females of all strains, but these *Md-dsx*^{M} signals were considerably weaker than those found in males.

Males of the M^{I} and F^{man} strains express dsx^{M} as well as dsx^{F}. Slight differences can be observed in the intensity of the signals; especially the F^{man} males seem to produce very little dsx^{F} transcripts. In contrast to the M^{I} strain, where YP can be detected in 80% of the males, only 4% to 40% of the F^{man} males synthesize YP. At the time when the RT-PCR analysis was performed, the percentage of F^{man} males that produced YP was at its minimum. This correlation of YP and dsx^{F} levels further supports our hypothesis that presence of dsx^{F} transcripts, and consequently of DSX^{F} protein, is responsible for YP production in *Musca* males.

Discussion

Earlier reports on yolk protein synthesis in *Musca domestica* have suggested that the YP genes are regulated entirely by hormonal activity, namely by a sex-specific concentration of ecdysteroids. However, some of the reported results did not fit into this model and suggested that additional factors must be involved in the regulation of YP production. A possible candidate for an additional factor is the product of the Musca *dsx* gene, Md-DSX. *Md-dsx* is a homologue of the Drosophila *dsx* gene and is, as *Dm-dsx*, sex-specifically spliced to give rise to two different proteins, the male DSX^{M} and the female DSX^{F} (Hediger *et al.*, 2004). It is well known that the Drosophila *dsx* proteins directly regulate YP synthesis by binding to the enhancer of the yp genes in the fat body and thus enhancing the basal transcription in females (DSX^{F}) or repressing it in males (DSX^{M}) (Burtis *et al.*, 1991; Coschigano and Wensink, 1993; An and Wensink, 1995; Cho and Wensink, 1997). Putative *dsx* binding sites have been found in the enhancer region of *Mdyp1*, as well as a 10bp consensus sequence called ovarian enhancer, which is conserved between Drosophila, Calliphora and Musca (Tortiglione and Bownes, 1997). These authors also showed that Dm-DSX binds *in vitro* to these putative binding sites and that the *Mdyp1* enhancer can tissue-specifically regulate a reporter gene in Drosophila; however, sex-specificity was not conferred by this promoter.

Our studies suggest that, apart from ecdysteroids, *Md-dsx* is involved in regulating YP expression in Musca. The most important indication that ecdysteroids cannot be solely

responsible for YP synthesis is the observation that YP producing Musca males do not have elevated ecdysteroid titres in their hemolymph. Instead, female *dsx* transcripts can be found in YP producing males, and the intensity of the signal correlates with the percentage of males synthesizing YP. In the F^{man} strain, where only about 4-40% of the males are YP positive, the signal is much weaker than in males of the M^{I} strain (80% YP). Thus, a common characteristic of males of the "YP strains" is a low ecdysteroid titre and presence of dsx^{F} transcripts.

In all Musca strains examined in this work, males produce only two YP, namely MDYP1 and MDYP3, whereas in females, two or three YP can be found, depending on the oocyte stage and on the corresponding ecdysteroid titre. A possible explanation for this fact is that MDYP2 is synthesized in the ovaries, and therefore cannot be produced by males. Since the *Mdyp2* transcript (White and Bownes, 1997) and the MDYP2 protein (this work, Fig. 3a) are also present in the fat body of females, this notion must be rejected. The only remaining explanation for the lack of MDYP2 in males is that expression of this YP requires high levels of ecdysteroids, as they only occur in females. In males, such ecdysteroid titres can only be generated experimentally by injection of 20E. This hypothesis is further supported by the finding that YP induction in males by injection of 20E is also dose-dependent; expression of *Mdyp2* requires a dose about 100 times higher than needed for the induction of *Mdyp1* and *Mdyp3*.

An explanation for the misregulation of *Mdyp1* and *Mdyp3,* but not *Mdyp2,* in the M^{I} and F^{man} strain is the possibility that a mutation occurred in *Mdyp1* and in *Mdyp3*. This is unlikely based on the following reasons: First, the two "YP strains" used in our experiments have arisen independently. Second, if M^{I} is combined with M^{III}, a strong *M* factor on chromosome 3, YP synthesis is completely abolished (Schmidt *et al.*, 1997b). The same is true if the dose of *F* in M^{I} males is reduced by combining M^{I} with one copy of the hypomorphic allele F^{man}, that is, in males with the genotype M^{I} /+; F^{man} /F^{+} (data not shown).

Interestingly, the homology on the protein level is higher between *Mdyp1* and *Mdyp3* than between *Mdyp2* and either *Mdyp1* or *Mdyp3* (White and Bownes, 1997). It seems that the

similarity does not only apply to the proteins themselves, but also extends to the control of YP expression, as *Mdyp1* and *Mdyp3* are always co-expressed. Some parallels to Drosophila can be found in that *Dmyp1* and *Dmyp2* share a common enhancer and are thus regulated simultaneously, whereas *Dmyp3* is located some 100kb away and contains different regulatory elements (Hung and Wensink, 1983). It has been speculated that *Dmyp1* and *Dmyp2* have arisen from a single ancestor YP gene by duplication; the same might be true for the Musca genes *Mdyp1* and *Mdyp3* (White and Bownes, 1997). Unfortunately, we do not know where in the Musca genome the *Mdyp* genes are positioned and how they are organized. However, a conserved 10bp ovarian enhancer element was only found in the enhancer regions of *Mdyp1* and *Mdyp3,* but not in that of *Mdyp2* (Tortiglione and Bownes, 1997; C.S., unpublished results). This also points to a different control mechanism and, possibly, separate evolution of *Mdyp2*.

Based on our results, we propose the following model of YP expression in *Musca domestica* (Fig. 7): The YP genes are controlled by the combined action of Md-DSX^F and ecdysteroids. In females, binding of Md-DSX^F to the enhancer region of *Mdyp1* and *Mdyp3* confers increased sensitivity to ecdysteroids. The relatively low level of ecdysteroids in pre- and postvitellogenic females is sufficient to drive expression of *Mdyp1* and *Mdyp3*. *Mdyp2*, however, requires a higher ecdysteroid titre to become expressed. Hence, only vitellogenic females with a high ecdysteroid titre are able to produce MDYP2. Md-DSX^M, which is also present in females, cannot repress the YP expression, as its concentration is much lower than that of DSX^F.

In males, Md-DSX^F is not present, and the ecdysteroid concentration is well below 10pg/µl. Under these conditions, none of the *Mdyp* genes can be activated. However, when males produce small amounts of Md-DSX^F, its binding to the enhancer of *Mdyp1* and *Mdyp3* can drive weak transcription of these genes, in spite of the low ecdysteroid level. *Mdyp2* cannot be activated since it requires a high ecdysteroid concentration that is never reached in males. Whether the presence of Md-DSX^M exerts a repressing function on the YP genes in Musca males, as Dm-DSX^M does in Drosophila, is not known.

Expression of all Mdyp genes can be induced in males without Md-DSX^F when sufficiently high doses of 20E are injected. The threshold for induction of *Mdyp2* again is much higher than those of *Mdyp1* and *Mdyp3*.

All together, the regulation of the *yp* genes in Musca and Drosophila shows parallels as well as differences. In both species, DSX proteins are involved in the control of YP expression in the fat body, and injection of 20E can stimulate YP production. In Musca, the key contribution comes from the ecdysteroids; they are responsible for the cycling of YP levels in females. Binding of Md-DSX^F alone merely leads to a sensitization of the *Mdyp* genes to ecdysteroids and to a weak basal YP synthesis in animals with a low ecdysteroid titre. In Drosophila, the *dsx* proteins are the key factors responsible for activation (DSX^F) or repression (DSX^M) of the *yp* genes. However, 20E can also induce *yp* gene transcription in Drosophila males (Bownes *et al.*, 1983; Shirk *et al.*, 1983), hence the corresponding binding sites must be present in the *Dmyp* genes (Bownes *et al.,* 1996), but it is not clear if they are necessary for controlling YP expression in the normal wildtype situation.

We propose that the *yp* genes were initially controlled by ecdysteroids produced by the ovaries to coordinate the availability of yolk with the maturation of oocytes. Later, *dsx* was recruited as an additional factor to implement differential responsiveness to ecdysteroids in male and female fat bodies. In species with continuous egg maturation and YP synthesis, *dsx* finally replaced the ecdysteroids as a regulator of YP synthesis in the fat body. In species like Musca, however, where egg production and YP expression are cyclic and need to be synchronized, ecdysteroids kept their role as the main controlling agents of YP production. Nevertheless, the *Mdyp* genes acquired some responsiveness to the action of the *dsx* proteins, which facilitates their activation by ecdysteroids in females. *Mdyp2*, which seems to be less responsive to DSX^F and needs a higher ecdysteroid titre to be activated, would then represent the more ancient mode of YP expression control.

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Captions

Figure 1: Correlation of ecdysteroid and YP concentration in the course of oocyte development. Stages 2 – 3: previtellogenic, stages 4 – 8: vitellogenic, stages 9 – 10: postvitellogenic stages. Summarized results from Adams and Filipi (1983) and Adams *et al.* (1985).

Figure 2: Sex determination in *Musca domestica.* *M* – male determining primary signal, located on the Y chromosome (standard strains) or on any of the five autosomes; *F* – female determining key gene (chromosome IV); *dsx*, *doublesex*, the terminal regulator of the sex-determining cascade.

Figure 3: (a-c) Western and Northern blot analysis of YP in hemolymph, fat body and ovaries of individual animals. m, marker **(a)** Western blot of YP in hemolymph (h), fat body (fb) and ovaries (ov) of males and females of a standard strain; presence of YP in female hemolymph depends on the oocyte stage (st.). **(b)** Western blot of YP in hemolymph (3 individuals showing different amounts of YP) and fat body of males of the M^{I} strain (left panel); YP in hemolymph, fat body and ovaries of females of the M^{I} strain (middle panel); YP in hemolymph of F^{man} males (right panel). Animals were 4d old when the samples were taken. **(c)** 1µg mRNA of pooled samples of standard females (left panel), standard males (right panel) and M^{I} males (middle panel) were analyzed for Mdyp transcripts by northern blotting.

Figure 4: YP induction by 20-hydroxy-ecdysone (20E) in males. Hemolymph of 4d old single males was analyzed for YP synthesis 24h after injection of 20E by western blotting (1µg, 10ng: amount of 20E injected).

Table 1: Ecdysteroid concentration in YP producing males. Pooled hemolymph samples of flies were analyzed for ecdysteroids by radio-immuno assay.

Figure 5: Male and female splice variants of Musca *dsx*. The male transcript consists of the exons 2, 3, m and 5; the female transcript contains exons 2, 3 and 4 (Hediger *et al.*, 2004). Arrows: Primers used for RT-PCR analysis of *dsx* transcripts.

Figure 6: Presence of dsx^{M} and dsx^{F} transcripts in males and females of standard and YP strains. 0.5µg mRNA of pooled samples was analyzed for sex-specific *dsx* transcripts by RT-PCR (primers see Fig. 5). 5µl samples were taken after 24, 27, 30 and 39 PCR cycles to estimate transcript abundance. *Sxl* transcripts served as a control for mRNA quality and abundance.

Figure 7: Model of YP expression in *Musca domestica*. Ecdysteroids, bound to their receptor (EcR), and DSX proteins act together to sex- and stage-specifically regulate YP synthesis. *Mdyp1* and *Mdyp3* are taken together since they are always coexpressed. Saturation of the red colour indicates the level of transcription. DRE, *dsx* responsive element (*dsx* binding sites); EcRE, ecdysteroid responsive element. The EcRE could not yet be located in the Mdyp genes; it is not clear whether they are upstream or downstream of the coding region.

Figures and Tables

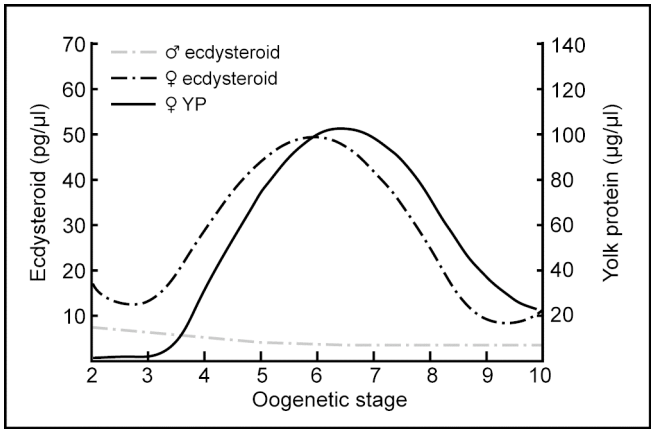

Figure 1

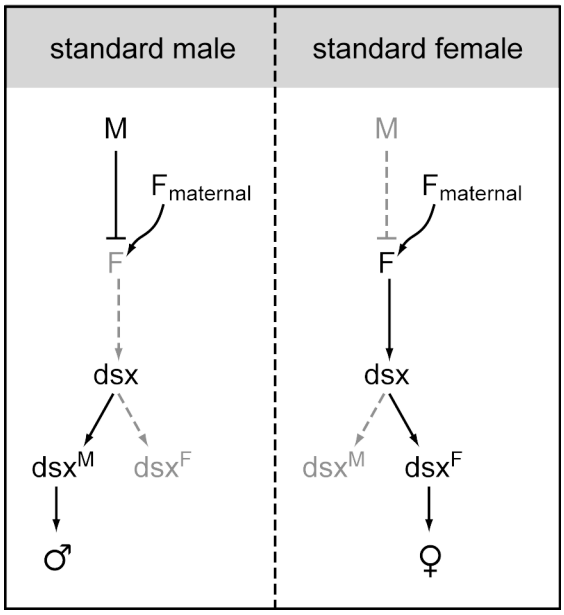

Figure 2

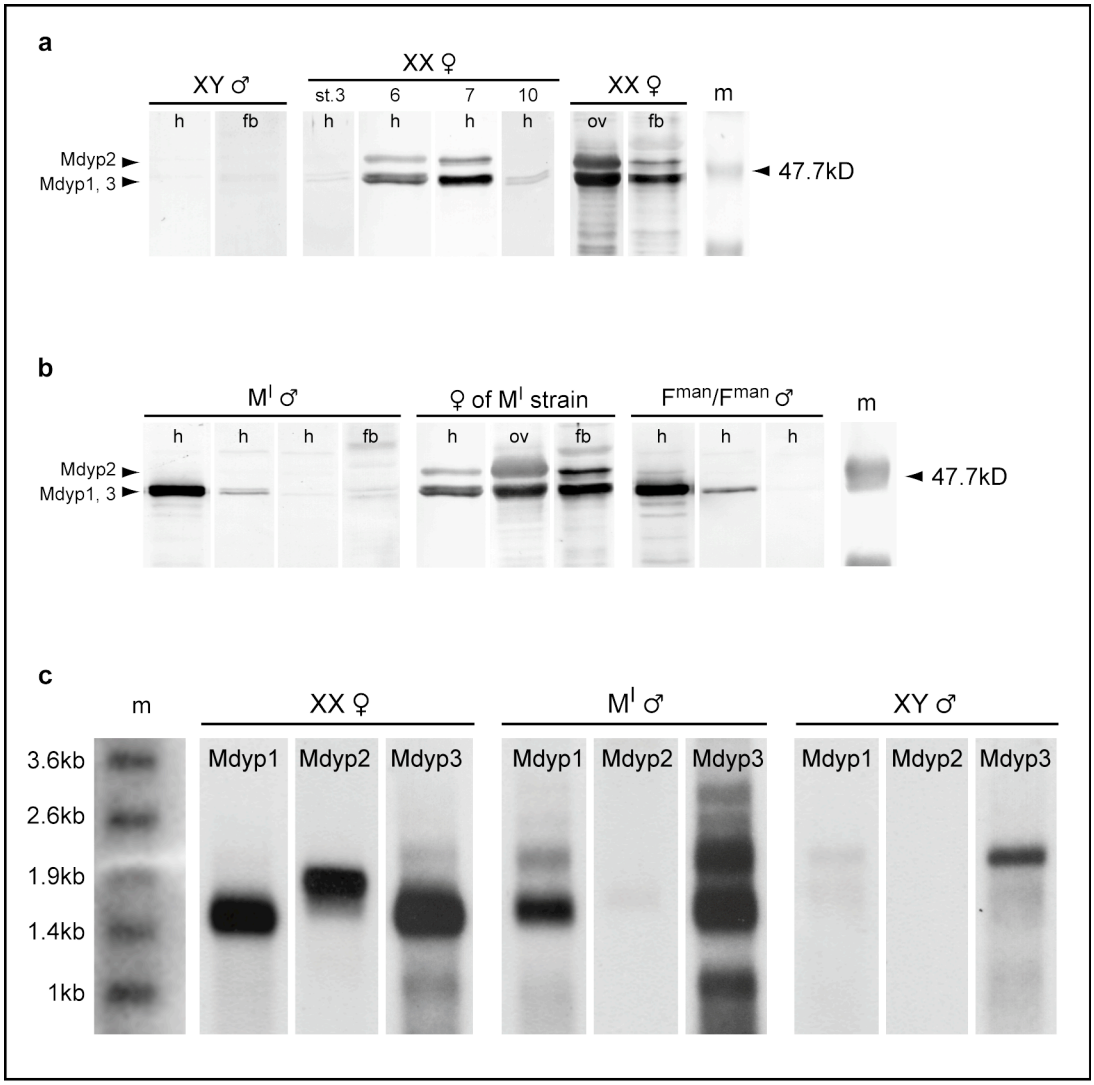

Figure 3

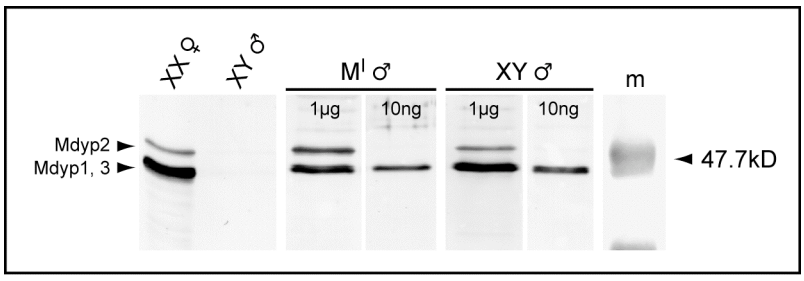

Figure 4

	Ecdysteroid concentration (pg/µl)			
Fly strain	females	(n)	males	(n)
Standard XX / XY	28.5	(98)	7.5	(200)
M^{I}	18.8	(150)	5.1	(150)
F^{man}	16.2	(118)	8.2	(258)

Table 1

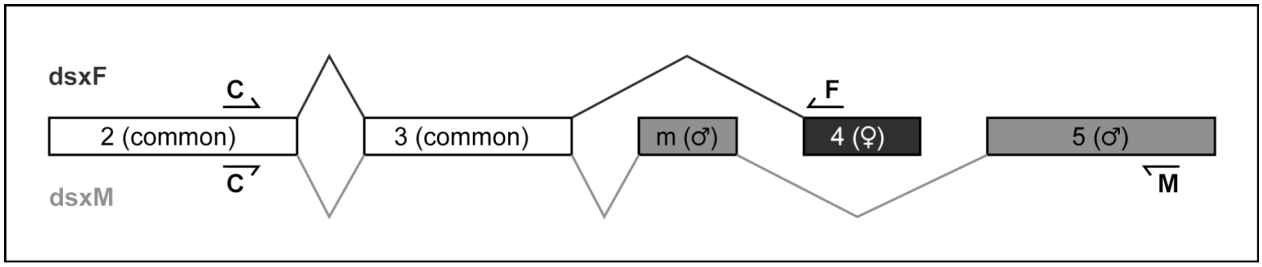

Figure 5

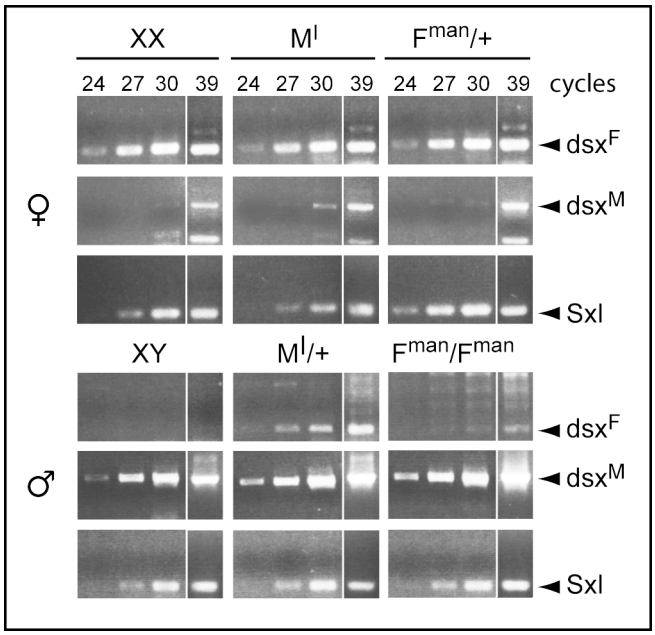

Figure 6

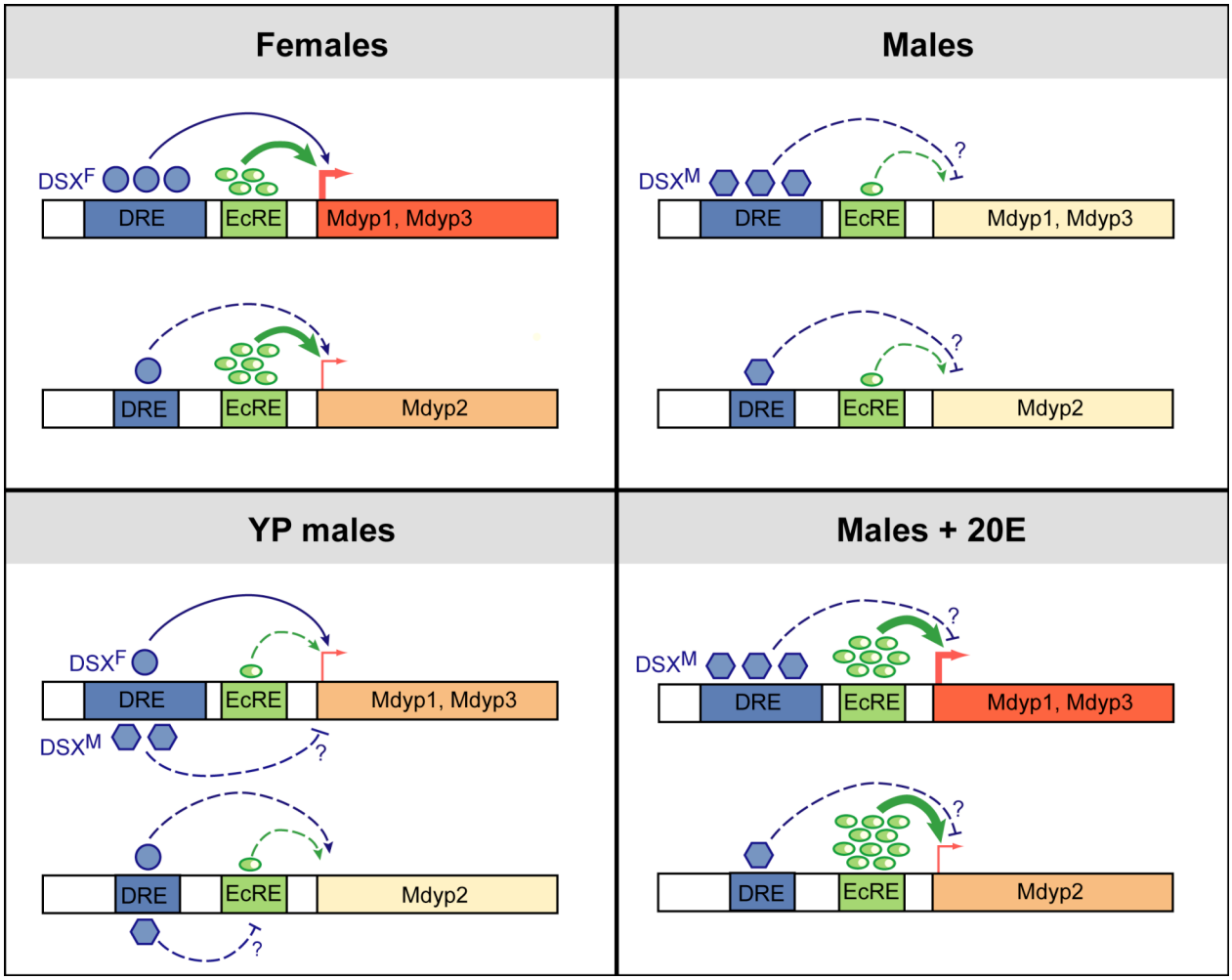
Females
DSXF
DRE
EcRE
Mdyp1, Mdyp3
DRE
EcRE
Mdyp2
Males
DSXM
?
DRE
EcRE
Mdyp1, Mdyp3
?
DRE
EcRE
Mdyp2
YP males
DSXF
DRE
EcRE
Mdyp1, Mdyp3
DSXM
?
DRE
EcRE
Mdyp2
?
Males + 20E
DSXM
?
DRE
EcRE
Mdyp1, Mdyp3
?
DRE
EcRE
Mdyp2

Figure 7

5 Discussion

5.1 *dsx*: a common terminal regulator of sexual differentiation in insects?

We have identified a *dsx* homologue in *Musca domestica* and shown that its structure and function are conserved. *dsx* homologues have also been found in other dipteran species. In all these species, including Musca and Drosophila, *dsx* is differentially spliced to give rise to a male- and a female-specific product; the male splice is used as the default variant, with the exception of Bombyx, where the female splice seems to be used by default (SUZUKI *et al.*, 2001).

The protein sequences of the *dsx* homologues are well conserved (Fig. 6). The male and the female proteins share a common C-terminus but differ at the N-terminal part. The common C-terminal part of the protein contains a conserved domain, the OD1 (oligomerization domain 1) or DM domain. This DNA binding domain forms an atypical zinc finger (BAKER *et al.*, 1989; ERDMAN and BURTIS, 1993); thus, male and female protein forms will bind to the same DNA sequences. The common part of the DSX protein contains another oligomerization domain, OD2; the second part of the OD2 domain is located in female specific part of the protein. OD2 is thought to be responsible for protein-protein interaction (AN *et al.*, 1996), hence the male and female DSX proteins will interact with different proteins. Consequently, the OD2 domain will enable the male and female protein isoforms, DSX^M and DSX^F, to exert different, sex-specific functions by recruiting different co-factors.

The structure of male and female specific mRNA reflects the protein structure: The 5' end of the mRNA is the same in both sexes, whereas the 3' ends consist of different, sex-specific exons. Again, the only exception is Bombyx, where the 5' and 3' ends of the mRNA in males and females are identical; female mRNA has two additional, female-specific exons between the common ones. The second female exon, exon 4, contains a stop codon, whereas in males, a stop in exon 5 is used for the termination of translation; therefore, the DSX^F and DSX^M proteins in Bombyx differ at their N-termini, though the 3' ends of the male and female specific mRNA are the same (SUZUKI *et al.*, 2001).

In Drosophila, the splice acceptor site of the female exon 4 is suboptimal as the consensus polypyrimidine sequence (Y_nNYAG) upstream of it is interrupted by several purine nucleotides. Female-specific splicing requires the binding of the TRA/TRA2 protein complex to a cis-acting splice enhancer site (HEDLEY and MANIATIS, 1991; HERTEL *et al.*, 1996; RYNER and BAKER, 1991; TIAN and MANIATIS, 1993); this site consists of six 13nt repeats called *dsx*-repeat elements (dsxRE; BURTIS and BAKER, 1989; INOUE *et al.*, 1992) and a purine rich element (PRE; LYNCH and MANIATIS, 1995) located in the 3' UTR of exon 4.

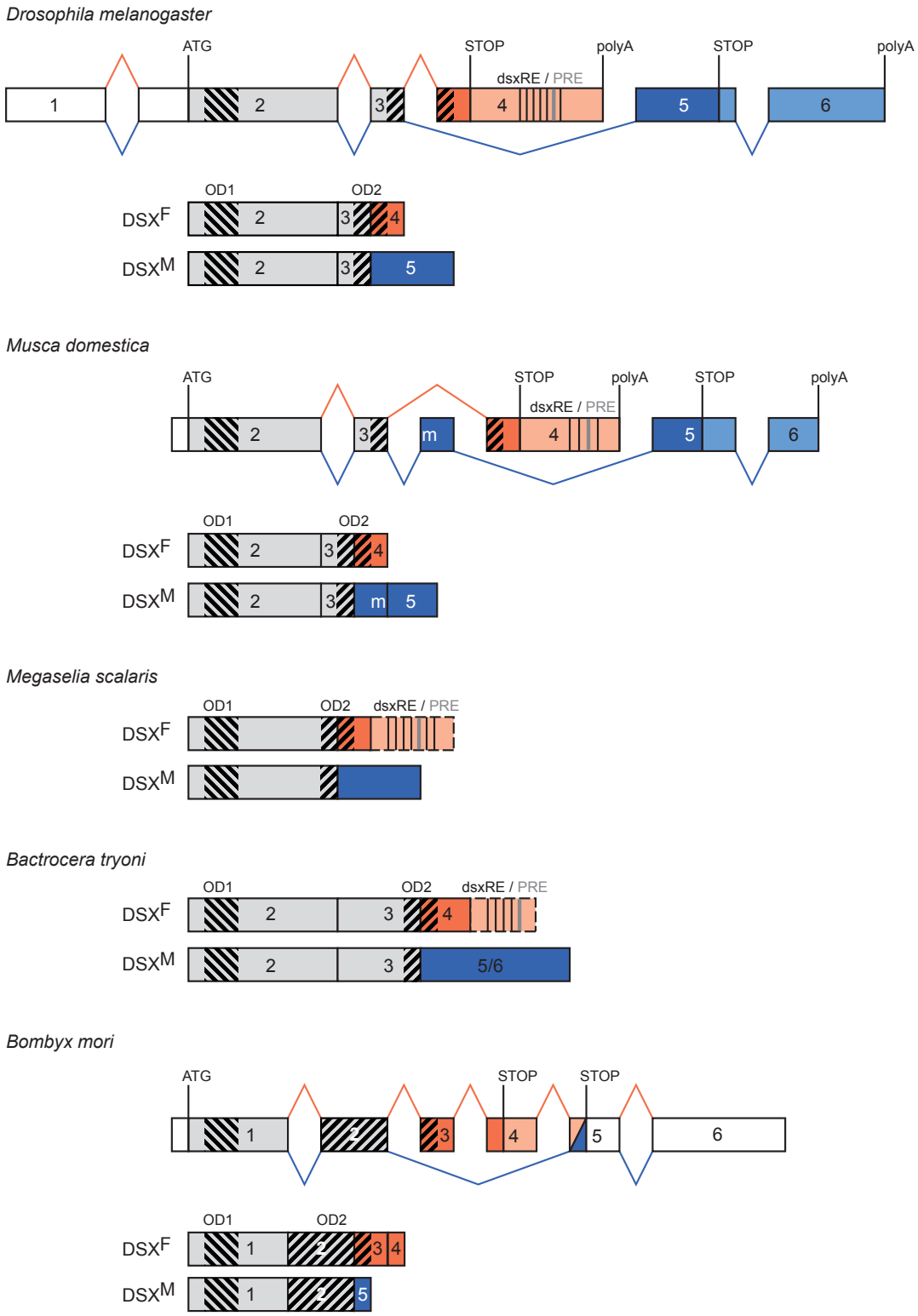

Figure 6: *dsx* homologues and their sex-specific splice variants in different insect species. dsxRE – *dsx* repeat elements (black lines); PRE – purin rich enhancer (grey lines); OD1 – oligomerization domain 1, the DNA binding domain; OD2 – oligomerization domain 2, for protein-protein interactions; dark red – female coding regions; light red – female 3' UTRs; dark blue – male coding region; light blue – male 3' UTRs; grey: common coding region; white – common 5' UTRs

In Drosophila, Megaselia, Bactrocera and Musca, the exon-intron structure of *dsx* is well conserved. In all these species, the male splice is the default variant, and the splice acceptor sites of upstream of the female exon were found to be suboptimal. Consequently, dsxREs and purin rich elements could be identified in all these *dsx* homologues, showing 80-100% identity compared to the Drosophila repeats. Except for Bactrocera, where the PRE is located downstream of the four dsxREs, the position of the dsxREs and the PREs are also conserved (KUHN *et al.*, 2000, SHEARMAN and FROMMER, 1996). In Musca, the situation is somewhat more complex due to the presence of the male exon m, since it has to be selectively repressed in females (HEDIGER *et al.*, 2004; see section 4).

The Bombyx *dsx* gene, in contrast, has a good splice acceptor site upstream of the female exon, and it also lacks dsxREs and a PRE. These findings concur with the fact that the female splice is the default in Bombyx (SUZUKI *et al.*, 2001), meaning that there is no need for a female-specific splicing enhancer. It seems thus likely that the upstream regulatory elements of *Bm-dsx* are divergent; SUZUKI *et al.* proposed that in the silk moth, the female exon is repressed in males by an unknown factor. Though protein structure and function of *dsx* are conserved between the lepidopteran species Bombyx and the dipteran species Drosophila, Megaselia, Bactrocera and Musca, the splice regulation of *dsx* in Bombyx seems to be divergent. This concurs with the hypothesis of WILKINS (1995), proposing that sex determination cascades evolved from bottom to top. The most conserved genes would thus be found at the bottom of the cascade, while those at the top diverged after speciation.

5.2 Regulation of *dsx*

Given that we found putative TRA/TRA2 binding sites in *Md-dsx*, we looked for homologues of the Drosophila upstream regulators *tra* and *tra2*. The gene *Md-tra2* could be identified by sequence homology (BURGHARDT *et al.*, in press). *Md-tra2* shows the following characteristics, which correspond to those of Drosophila *tra2*:

1. *Md-tra2* is expressed in both sexes;
2. *Md-tra2* is essential for female development;
3. *Md-tra2* acts upstream of *Md-dsx*.

Since *Md-tra2* is expressed in both sexes, it can only be a necessary co-factor for female development; another gene, which cooperates with *Md-tra2*, must be responsible for the female specific functions of *Md-tra2*. We propose that, in analogy to Drosophila, the Musca *tra* homologue is this factor, and that Musca *tra* corresponds to the genetically identified female-determining *F* gene (M. Hediger, unpublished results). According to our model, in females, F (Md-TRA?) protein forms a complex with Md-TRA2 and promotes

the female splicing of *dsx* by activating the weak splice acceptor site upstream of exon 4. In males, where no F (Md-TRA?) protein is present, Md-TRA2 alone will not be able to activate this splice site, and *dsx* will thus be spliced in the default male mode.

Zygotic activation of *F* (*Md-tra*?) requires the presence of maternally provided F product (DÜBENDORFER and HEDIGER, 1998). The necessity for maternal F product together with the fact that injection of *Md-tra2* dsRNA in early embryos leads to complete sex-reversal of genetically female animals, lead us to hypothesize that *F* (*Md-tra*?) is positively autoregulated as it has been proposed for the *Ceratitis capitata transformer* gene (PANE *et al.*, 2002): An Md-TRA/Md-TRA2 complex would thus not only influence the splicing of the downstream gene *Md-dsx*, but also be responsible for the female-specific splicing of the *F* mRNA. Female splicing of *F* mRNA would thus only be possible in the presence of its own protein. If maternal F product is lacking, or the autoregulatory loop of *F* is interrupted in early embryogenesis by RNAi against *Md-tra2*, production of female F cannot be maintained, and the affected animal will develop into a male (BURGHARDT *et al.*, in press). Presence of a male-determining *M* factor of course has the same effect; how the *M* factor represses production of female *F* (*Md-tra*?) is not known; it could act as a transcriptional repressor of F or interfere with the splice regulatory activity of *F* or *Md-tra2*. In Drosophila, the task of memorizing the sexual fate of a cell has been delegated one step further up in the hierarchy to the level of *Sex-lethal*, supporting the hypothesis that the gene *Sex-lethal* has taken over this task from *tra* and *tra2*.

5.3 Regulation of yolk protein synthesis: a common mechanism in insects?

Yolk protein synthesis in most insect species examined so far seems to be regulated by hormones, especially juvenile hormone and ecdysteroids. In contrast, *Drosophila melanogaster* uses the transcription factor *dsx* to regulate YP synthesis: The female splice variant, DSX^F, enhances the basal transcription level in females, whereas the male splice form, DSX^M, represses it in males (reviewed in BOWNES, 1994). The obvious question arises whether regulation of YP synthesis by *dsx* is an exception or whether this gene plays a direct role in controlling YP expression in other insect species. *Musca domestica*, the common housefly, apparently uses the widespread mechanism based on hormonal control. Ecdysteroid titers in females correlate with the amount of YP synthesized, and injection of 20-hydroxy-ecdysone induces YP production in males (ADAMS and FILIPI, 1983; ADAMS *et al.*, 1985; AGUI *et al.*, 1985; ADAMS *et al.*, 1989). However, there is evidence that other factors are involved. First, males do not react as well to injection of 20E as females. Second, ovaryectomized females continue to express YP (AGUI *et al.*, 1991).

Putative *dsx* binding sites were identified in the enhancer region of *Mdyp1*, and Drosophila DSX was found to bind to this region (TORTIGLIONE and BOWNES, 1997).

This lead us to hypothesize that a Musca homologue of *dsx* exists that is required for the control of YP expression in Musca. We were able to identify this homologue in Musca. As in Drosophila, it is differentially spliced to give rise to sex-specific protein variants with opposing activities (HEDIGER *et al.*, 2004; see section 4)

Our results show that, apart from ecdysteroids, *dsx* may play a role in the regulation of YP production in Musca:

1. Ecdysteroid titres are not elevated in Musca males that synthesize small amounts of YP.

2. Md-DSX^F, the female protein variant of *Md-dsx* is expressed in these males.

3. Ectopic expression of *Md-dsx*^{F} can induce weak YP expression in males (HEDIGER *et al.*, 2004, see section 4).

In the silkworm *Bombyx mori*, a *dsx* homologue was recently identified (OHBAYASHI *et al.*, 2000). Ectopic expression of its female splice form leads to induction of weak vitellogenin (Vg) synthesis in Bombyx males (SUZUKI *et al.*, 2003). The lepidopteran species Bombyx is only distantly related to the dipterans Drosophila and Musca, and its vitellogenin genes are not homologous to the *yp* genes (ROMANS *et al.*, 1995); nevertheless, it seems that their control by *dsx* is conserved. A similar situation can be found with regard to ecdysteroids: Both *Vg* (e.g. Aëdes) and *yp* genes can be regulated by hormones (e.g. Musca).

Until now, it seemed that YP synthesis is controlled either cell-autonomously by transcription factors such as *dsx* (e.g. Drosophila), or non-autonomously by hormones (e.g. Musca). A plausible explanation for the existence of different mechanisms would be that the type of regulation depends on how oogenesis occurs. Species with continuous oocyte development, as Drosophila, require a constant availability of YP and thus use transcription factors to activate or repress YP synthesis in females or males, respectively, whereas species having a cyclic oogenesis use hormones to synchronize oocyte development with the level of YP expression, as is the case in Musca. However, why would the Drosophila *yp* genes then respond to ecdysteroids (which they do, as has been shown by injection of 20E into males), if *dsx* is the one and only player in this game? This question also leads straight to another one, namely if there really exist two different, separate systems for regulation of YP synthesis in different species. The results of our studies with *Musca domestica* indicate that these two mechanisms – transcription factors and hormones – can control YP synthesis in one species by acting in concert.

Still, even if transcription factors and hormones act together in the same species, why is it necessary to make use of two mechanisms to control YP expression? To answer this question, we have to consider that YP synthesis does not only have to be sex-specific, but

also tissue-specific, stage-specific and synchronized with oogenesis and that it also needs to respond to environmental changes. For example, it does not make much sense to produce YP if there is no suitable substrate available for oviposition, or if the nutritional status of the female is very poor. We thus propose a novel model for the control of YP synthesis in insects (Fig 7A):

- DSX^F and DSX^M are responsible for modulating the responsiveness of the YP genes to activation by hormones;
- additional transcription factors confer tissue-specificity, for instance the DmC/EBP transcription factor in Drosophila or the β-FTZ-F1 transcription factor in *Aëdes aegypti*, which acts as a competence factor for Vg expression in the fat body;
- hormones such as ecdysteroids are used to synchronize YP synthesis with ovarian vitellogenesis;
- hormones such as ecdysteroids or juvenoids are responsible to adjust YP synthesis to environmental conditions.

All phenomena associated with YP synthesis in Drosophila and Musca can be explained by this model. In Drosophila females (Fig. 7B), DSX^F directly activates transcription of the *yp* genes, even when the ecdysteroid titer is low. YP production is stopped when females are starved. Since expression of $DSX^F$is not affected by malnutrition (SONDERGAARD *et al.*, 1995), other factors must be responsible for this halt in YP expression. Possibly, the ecdysteroid titer in these females drops to such a low level that YP synthesis cannot be maintained, not even in the presence of DSX^F. In males, DSX^M represses transcription of the *yp* genes and also decreases their sensitivity to activation by ecdysteroids. Nevertheless, if a sufficiently high dose of 20E is injected into males, the hormone can override the repressing action of DSX^M.

In Musca, Md-DSX^F and Md-DSX^M act in a similar fashion as they do in Drosophila, though their influence may be less distinct (Fig. 7C). Hormones appear to play a far more prominent role in Musca. Since oocyte development in Musca is synchronous and occurs in cycles, it is essential to adjust YP production to ovarian vitellogenesis; this is achieved by the secretion of ecdysteroids by the ovary according to the stage of oocyte development.

Thus, if we only cast a quick glance at how YP expression may be regulated, we will find ecdysteroids in Musca and *dsx* in Drosophila; but if we take a closer look, we notice that they actually employ both mechanisms, though at a different degree. We can regard these two species as two extrema in the varying contribution of *dsx* and hormones to the control of YP synthesis. Other species may be situated somewhere inbetween them.

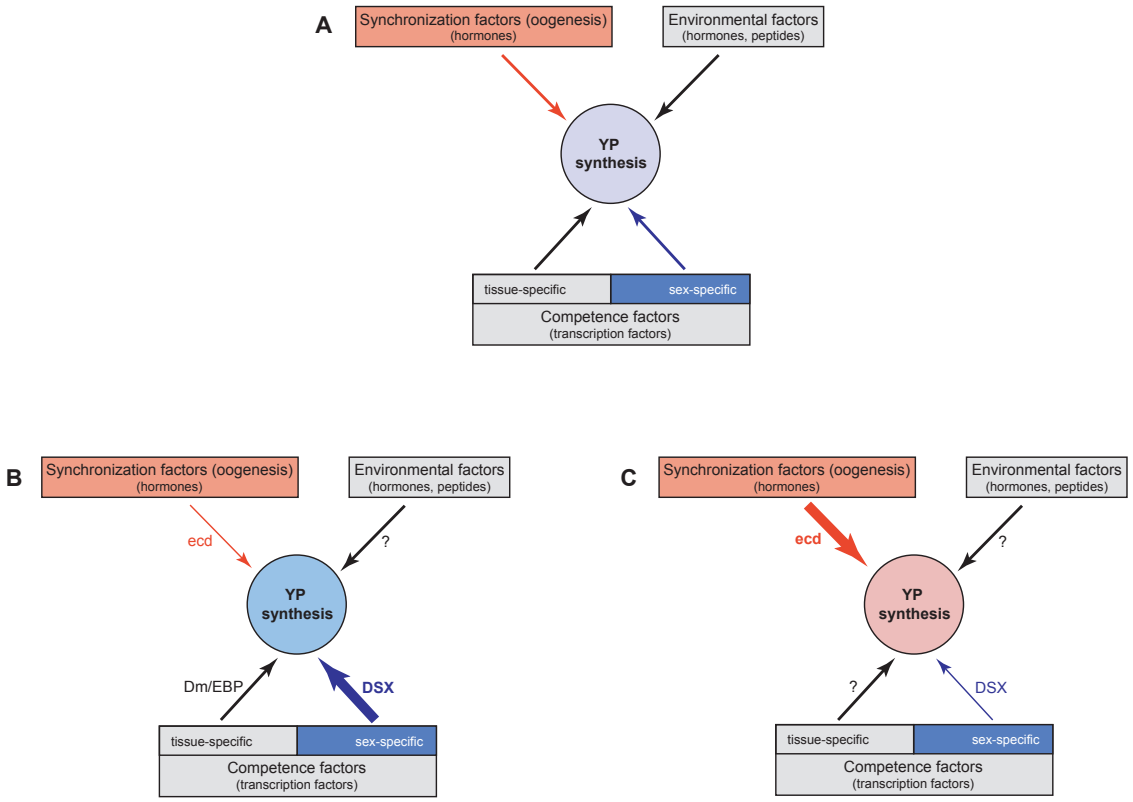

Figure 7: Model of the regulation of YP synthesis in insects. Weight of arrows indicates the contribution of the factors. A – general model; B – *Drosophila melanogaster*; C – *Musca domestica*. Question marks indicate that the nature and contribution of this factor are not known. ecd – ecdysteroids

At least three species are now known that use *dsx* or its homologues in controlling YP expression, Drosophila, Musca and Bombyx (SUZUKI *et al.*, 2003). No *dsx* homologue has been found so far in species like Locusta, Blatella, Lucilia or Sarcophaga, where the synthesis of yolk proteins is apparently controlled by ecdysteroids or juvenile hormone. *dsx* homologues have been identified in Megaselia (SIEVERT *et al.*, 1997; Kuhn *et al.*, 2000), Anopheles (PANNUTI *et al.*, 2000), Ceratitis (Saccone *et al.*, 1996), and Bactrocera (SHEARMAN and FROMMER, 1996), but it is not known whether these homologues are involved in the control of YP synthesis in these species. However, since *dsx* is a highly conserved gene, it is very likely that it is also present in other insects, and that, if it is present, it also contributes to the regulation of yolk protein synthesis.

5.4 Model of the sex determination cascade in *Musca domestica*

Based on the newly identified Musca genes *Md-dsx* and *Md-tra2* and on the findings concerning the influence of *Md-dsx*on the control of YP expression, we developed a model of sex determination in Musca. In standard wildtype strains, the *M* factor on the Y chromosome completely represses the zygotic female-determining gene *F* (Fig. 8A). Though

maternal F product and maternal Md-TRA2 are present, F can not be activated, resulting in *Md-dsx* being spliced in the default male mode. No YP are synthesized since the ecdysteroid level is low, and no Md-DSX^F is present. Possibly, Md-DSX^M even exerts a repressing action on the *Mdyp* genes. The ovary is probably the main source for the ecdysteroids in Musca females, since the ecdysteroid concentration drops to a male-like level in ovaryectomized females (AGUI *et al.*, 1991). Where the male ecdysteroids are synthesized, and which might be additional sources in females, is not known.

When M is absent, maternal Md-TRA2 and F product allow the zygotic F to be activated (Fig. 8B). A protein complex of F and Md-TRA2 keeps the F gene active through an autoregulatory feedback and *Md-dsx* is spliced into the female mRNA. This female produces yolk proteins, since Md-DSX^F is present, and the ecdysteroid titre is high.

In the M^I strain, males carry a weak M factor on chromosome I (Fig. 8C). M^I is not able to completely repress zygotic F, allowing a small amount of F protein to be produced. This in turn will lead to some *Md-dsx* pre-mRNA to be spliced into the female mode (SIEGENTHALER *et al.*, see section 4). There is not enough Md-DSX^F for the animal to be morphologically feminized, but enough to allow some expression of YP. The ecdysteroid titer in these males remains as low as in wildtype males (SIEGENTHALER *et al.*, see section 4).

Two mutations of the F gene in Musca are known: F^D and F^{man}. In these strains, sex does not depend on the presence or absence of an M factor, but on the presence or absence of F^D or F^{man}. In F^{man} strains, there is no M factor (Fig. 8D). Male development occurs when a zygote is homozygous for the F^{man} allele. Since F^{man} is a hypomorphic allele (SCHMIDT *et al.*, 1997a), there is some residual activity of F, and, analogous to M^I males, these F^{man} males express Md-DSX^F (SIEGENTHALER *et al.*, see section 4) and, consequently, YP (SCHMIDT *et al.*, 1997a). Females of this strain are heterozygous F^{man}/F^+; the activity of one F^+ allele seems to be sufficient to support normal female development.

In F^D strains (Fig. 8E), all animals carrying the dominant F^D allele will develop into females, no matter if one or even several M factors are present. F^D does not depend on maternal F product to be active, suggesting that it does not depend on an autoregulatory activity. Also, the fact that F^D is resistant to repression by M suggests that M primarily disrupts the autoregulatory function of F. Animals of these strains which do not carry the F^D allele, but one or several M factors, develop into males. As in standard wildtype strains, M will repress the wildtype alleles of F (DÜBENDORFER and HEDIGER, 1998).

When we compare the sex determination cascade of Musca with the cascade in Drosophila, it is obvious that the lower part of the pathway is conserved, as proposed by WILKINS (1995): The terminal regulator genes, *dsx* and *Md-dsx* are structurally and

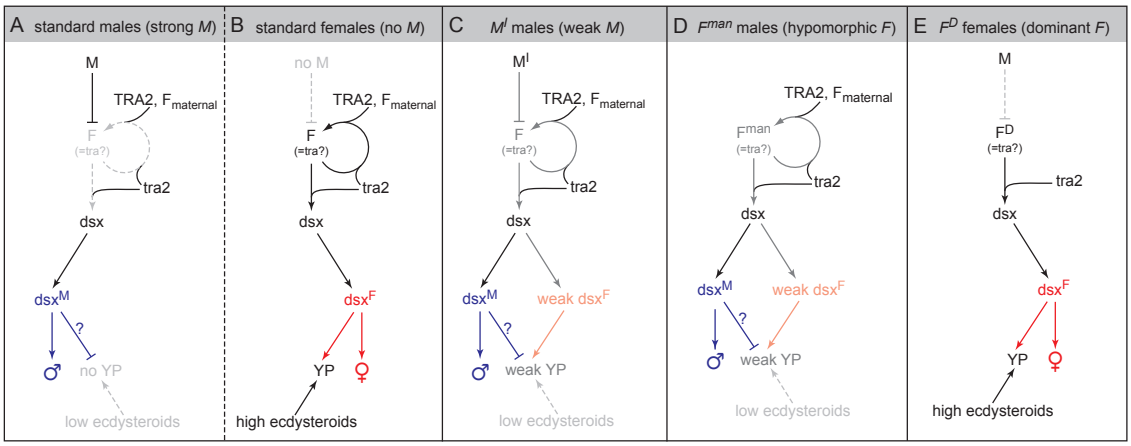

Figure 8: Model of the sex determination cascade in different Musca strains

functionally conserved, and so are the co-factors *tra2* and *Md-tra2* found one level higher up in the hierarchy. There also is good evidence that the Musca *F* gene is a *tra* homologue (M. Hediger, unpublished results). The homology, however, ends when we look at the upstream regulators of *tra* and *F*, respectively. In Drosophila, there is an additional step in the cascade, the gene *Sxl*, which is responsible for remembering the sexual fate, and upstream of *Sxl*, we finally find the primary signal – the X:A ratio. In Musca, the task of remembering the sexual fate is probably performed by *F*, and immediately upstream of *F* (*Md-tra?*), we find the primary signal, which is a single gene, the dominant male determining factor *M*. *Md-Sxl* plays no role in sex determination (MEISE *et al.*, 1998)

Additional work will be required to identify the missing components of the Musca sex determining cascade, especially the molecular nature of *M*. Comparing the sex determination mechanisms of the evolutionary old species Musca to the ones of other dipteran and non-dipteran insects will help us to understand the evolution of complex signalling pathways.

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A Curriculum vitae

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Publikationen

Hediger, M., Burghardt, G., **Siegenthaler, C.**, Buser, N., Hilfiker-Kleiner, D., Dübendorfer, A., Bopp, D. (2004)
Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*.
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Burghardt, G., Hediger, M., **Siegenthaler, C.**, Moser, M., Dübendorfer, A., Bopp, D.
The *transformer2* gene in *Musca domestica* is required for memorizing and executing the female pathway of development.
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Siegenthaler, C., Maròy, P., Hediger, M., Bopp, D., Dübendorfer, A.
Yolk protein synthesis in Musca domestica is controlled by ecdysteroids and *dsx* proteins.
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Dissertation

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Steroid hormones and transcription factors in yolk protein synthesis of *Musca domestica*

Leitung:
Prof. Andreas Dübendorfer und Dr. Daniel Bopp

Antragstellendes Fakultätsmitglied:
Prof. Ernst Hafen

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